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Biochimica et Biophysica Acta 1778 (2008) 313–323
Biochimica et Biophysica Acta BBA
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Glycyrrhetinic acid as inhibitor or amplifier of permeability transition in rat heart mitochondria

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Received 2 April 2007; received in revised form 31 August 2007; accepted 4 October 2007
Available online 16 October 2007

Abstract

Glycyrrhetinic acid (GE), a hydrolysis product of glycyrrhizic acid, one of the main constituents of licorice root, is able, depending on its concentration, to prevent or to induce the mitochondrial permeability transition (MPT) (a phenomenon related to oxidative stress) in rat heart mitochondria (RHM). In RHM, below a threshold concentration of 7.5 μM, GE prevents oxidative stress and MPT induced by supraphysiological Ca^{2+} concentrations. Above this concentration, GE induces oxidative stress by interacting with a Fe–S centre of Complex I, thus producing ROS, and amplifies the opening of the transition pore, once again induced by Ca^{2+}. GE also inhibits Ca^{2+} transport in RHM, thereby preventing the oxidative stress induced by the cation. However, the reduced amount of Ca^{2+} transported in the matrix is sufficient to predispose adenine nucleotide translocase for pore opening. Comparisons between observed results and the effects of GE in rat liver mitochondria (RLM), in which the drug induces only MPT without exhibiting any protective effect, confirm that it interacts in a different way with RHM, suggesting tissue specificity for its action. The concentration dependence of the opposite effects of GE, in RHM but not RLM, is most probably due to the existence of a different, more complex, pathway by means of which GE reaches its target. It follows that high GE concentrations are necessary to stimulate the oxidative stress capable of inducing MPT, because of the above effect, which prevents the interaction of low concentrations of GE with the Fe–S centre. The reported results also explain the mechanism of apoptosis induction by GE in cardiomyocytes.

Keywords: Glycyrrhetinic acid; Mitochondria; Permeability transition; Heart

1. Introduction

Glycyrrhetinic acid (GE) is the active aglycone of glycyrrhizin, a pentacyclic triterpene glycoside constituting one of the main components of licorice root. Licorice has long been known as a medicinal plant, and its history as a pharmacological remedy dates back far into the past [1]. Licorice possesses several biological and endocrine properties: in particular, it behaves as a mineralocorticoid, gluco- and antigluco-corticoid and has estrogenic and anti-estrogenic effects [1]. It has recently been used to treat viral infections such as hepatitis, HIV, and chronic fatigue [2–5]. *In vivo* investigations have demonstrated that GE induces programmed cell death, most probably by inhibiting the liver glucocorticoid metabolizing enzyme 11-hydroxysteroid dehydrogenase type I (11HSD1) [6,7]. However, it has also been reported that, in isolated rat liver mitochondria (RLM), GE is able to induce the phenomenon of mitochondrial permeability transition (MPT) [8] by means of oxidative stress [9], also suggesting the involvement of this substance in triggering the

Abbreviations: AdNT, adenine nucleotide translocase; AIF, apoptosis inducing factor; BHT, butylhydroxytoluene; BKA, bongkrekic acid; CsA, cyclosporin A; ER, endoplasmic reticulum; GE, glycyrrhetinic acid; MPT, mitochondrial permeability transition; PARP, poly (ADP-ribose) polymerase; RHM, rat heart mitochondria; RLM, rat liver mitochondria; ROS, reactive oxygen species; TPP^+, tetraphenylphosphonium; 11HSD, 11-hydroxysteroid dehydrogenase; $\Delta\Psi$, membrane potential

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doi:10.1016/j.bbamem.2007.10.008

pathway of mitochondrial-mediated apoptosis. The GE derivative, carbenoxolone, has effects similar to those of GE, although with less efficacy [10].

It is known that, *in vitro* and *in vivo*, GE has mineralocorticoid activity, both by blocking 11HSD type 2 and by binding to mineralocorticoid receptors [11]. In previous studies we showed that, in human mononuclear leukocytes, both GE and aldosterone can regulate the intracellular electrolyte concentration at lower levels [12] and, at higher concentrations, can induce oxidative stress *in vitro* and *in vivo* [13]. In particular, aldosterone is involved in the induction of fibrosis and hypertrophy in the heart.

Mitochondria are a source of reactive oxygen species (ROS), and decreased antioxidant activity may contribute to the onset of many heart diseases. In isolated cardiac myocytes, a slight increase in ROS results in a phenotype characterised by hypertrophy and fibrosis. ROS of mitochondrial origin can trigger apoptosis through increased MPT pore opening, release of cytochrome *c*, and activation of enzymes involved in apoptosis signalling [14,15].

Oxidative stress increases in myocardial failure and the formation of superoxide also increases in submitochondrial particles. Complex 1 activity is 50% lower, suggesting a block of the electron transport system [16].

The aim of this study was to determine the effects of GE in RHM in comparison with those observed in RLM [8,9], and to evaluate if heart mitochondria are involved in the mechanism of apoptosis induction by GE previously reported in cardiomyocytes [17].

2. Materials and methods

2.1. Chemicals

18β-Glycyrrhetinic acid was purchased from Sigma and dissolved in absolute ethanol. Mouse monoclonal antibody anti-cytochrome *c* was purchased from Pharmingen. All other reagents were of the highest purity commercially available.

2.2. Mitochondria preparation

Mitochondria were prepared from the hearts and livers of male Wistar rats weighing about 250 g. RHM were isolated in a buffer containing 300 mM sucrose, 5 mM HEPES (pH 7.5), 0.1% bovine serum albumin and 10 mM EDTA [18]; EDTA was omitted from the final washing solution. RLM were isolated by conventional differential centrifugation in a buffer containing 300 mM sucrose, 5 mM HEPES (pH 7.4), and 2 mM EGTA [19]; EGTA was omitted from the final washing solution. Protein content was measured by the biuret method, with bovine serum albumin as standard [20]. Isolated RHM and RLM were purified in a Ficoll discontinuous gradient as previously described [21]. Both preparations were examined by electron microscopy, and showed no microsomes or peroxisomes as contaminants. Respiratory control indexes (RCI) and the ADP/O ratio for RHM were 6.2 and 1.92, respectively; for RLM, the values were 5.7 and 1.87, respectively. Membrane potential ($\Delta\Psi$) values were 181 and 174 mV for RHM and RLM, respectively. These values of RCI, ADP/O and $\Delta\Psi$ are the means of a large number of assays.

2.3. Standard incubation procedures

RHM or RLM (1 mg prot./ml) were incubated in a water-jacketed cell at 20 °C. Experiments were carried at 20 °C, because the respiratory chain of isolated mitochondria at higher temperatures, e.g., 37 °C, operates at a high rate. As the amount of O_2 solved in *in vitro* conditions in the suspension medium is limited, anaerobiosis is normally reached in a few minutes, resulting in the bioenergetic collapse of mitochondria. The standard medium contained 250 mM sucrose, 10 mM HEPES (pH 7.4), 5 mM succinate, 1.25 μM rotenone, and 50 or 200 μM Ca^{2+} was added, as indicated in the figure legends. Variations and/or other additions are described in the legend of any single experiment. The control assays contained the same amount 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

RCI, ADP/O ratio and $\Delta\Psi$ were calculated as reported in Ref. [22].

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

Protein sulfydryl oxidation assay was performed with 5,5′-dithiobis(2-nitrobenzoic acid) at 412 nm in a Kontron Uvikon model 922 spectrophotometer, according to Santos et al. [23].

Reduced and oxidised glutathione (GSH and GSSG respectively) were determined according to Tietze [24].

The calcium ion content of the supernatant and its fluxes across the membrane were estimated by atomic absorption spectroscopy, on a Perkin-Elmer 110B spectrometer and an Ionetics Calcium STAT electrode.

2.5. Detection of cytochrome c and AIF release

Mitochondria (1 mg prot./ml) were incubated for the times indicated in the specific experiments at 20 °C, in standard medium, with the appropriate additions. The reaction mixtures were then centrifuged at 13,000 *g* for 10 min at 4 °C to obtain mitochondrial pellets. The supernatant fractions were further spun at 100,000 *g* for 15 min at 4 °C, to eliminate mitochondrial membrane fragments, and concentrated five times by ultrafiltration through Centrikon 10 membranes (Amicon) at 4 °C. Aliquots of 10 μl of the concentrated supernatants and mitochondrial pellets (25 μg) were subjected to SDS-PAGE with 15% (w/v) acrylamide vertical slab gel and analysed by Western blotting with mouse anti-cytochrome *c* and rabbit anti-AIF monoclonals antibodies (ICN Biotechnology), with an enhanced chemiluminescent detection system (ECL, Amersham Pharmacia Biotech).

2.6. Cardiomyocyte preparation and morphological evaluation of apoptosis

Cardiomyocytes were prepared by collagenase digestion as previously described [25], cultured for 24 h in DMEM supplemented with 10% calf serum, 10% equine serum and 1% penicillin/streptomycin, and subsequently incubated for 24 h in DMEM in the absence or presence of 5 and 10 μM GE. After washing and fixation, cardiomyocytes were incubated with Hoechst dye 33258 (8 μg/ml) in PBS (0.8% NaCl, 0.02% KCl, 0.115% Na_2HPO_4 and 0.2% KH_2PO_4, pH 7.2) for 5 min, washed with PBS and mounted with glycerol in phosphate-buffered saline. For each experimental observation, the percentage of apoptotic cells per 1000 cells was scored by fluorescent microscopy (Leica DMLB).

2.7. Cleavage of PARP

Cleavage of PARP was detected according to Gardini et al. [26]. In brief, at the end of the culture, cardiomyocytes were washed twice with PBS and collected with boiling loading buffer. After sonication and centrifugation, aliquots of the supernatant corresponding to 100 μg protein were analysed by SDS-PAGE on an 8% polyacrylamide gel. Standard protein markers were used for molecular weight calibration. After blotting, immunoreaction bands were detected by ECL.

3. Results

The apparent absorbance of an RHM suspension, incubated in standard medium and in the presence of 50 μM Ca^{2+}, did not

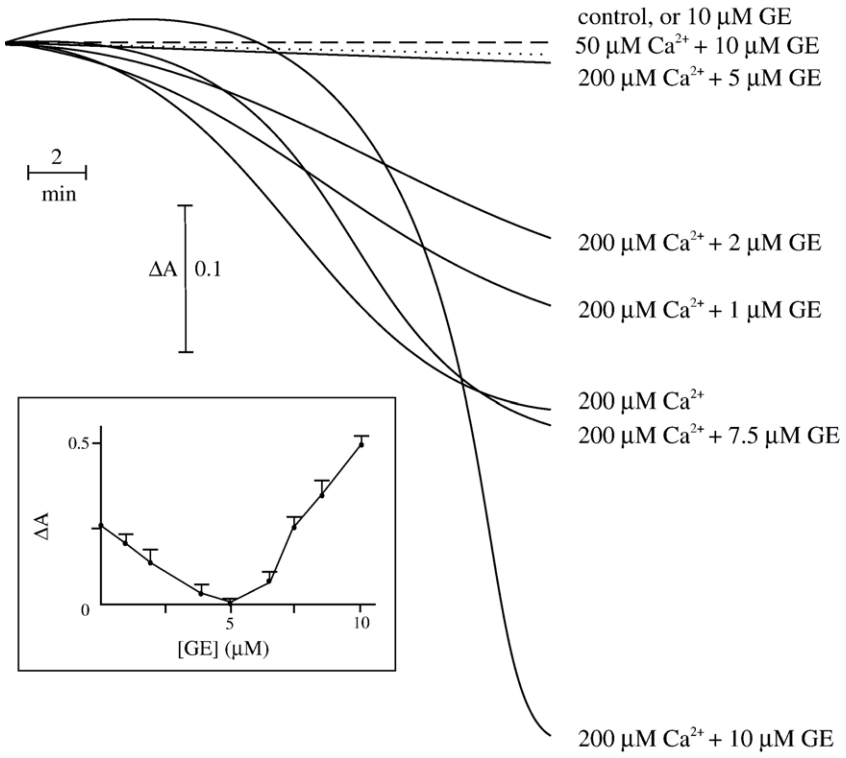


Fig. 1. Dose-dependent effect of GE on swelling of RHM induced by Ca^{2+}. RHM were incubated in standard medium supplemented with 50 μM Ca^{2+} (dotted line) or 200 μM Ca^{2+}, in conditions indicated in Materials and methods. Control curve is in absence of Ca^{2+} (dashed line). GE was present at concentrations indicated on side of curves. GE alone, at all concentrations gave same result as controls. Four other assays gave almost identical results. Downward deflections of traces recording apparent absorbance changes at 540 nm indicate mitochondrial swelling. Inset: dose-dependence by GE with 200 μM Ca^{2+}, calculated as maximum extent of ΔA after 20 min of incubation at every concentration indicated in plot. Mean values ± SD of four experiments.

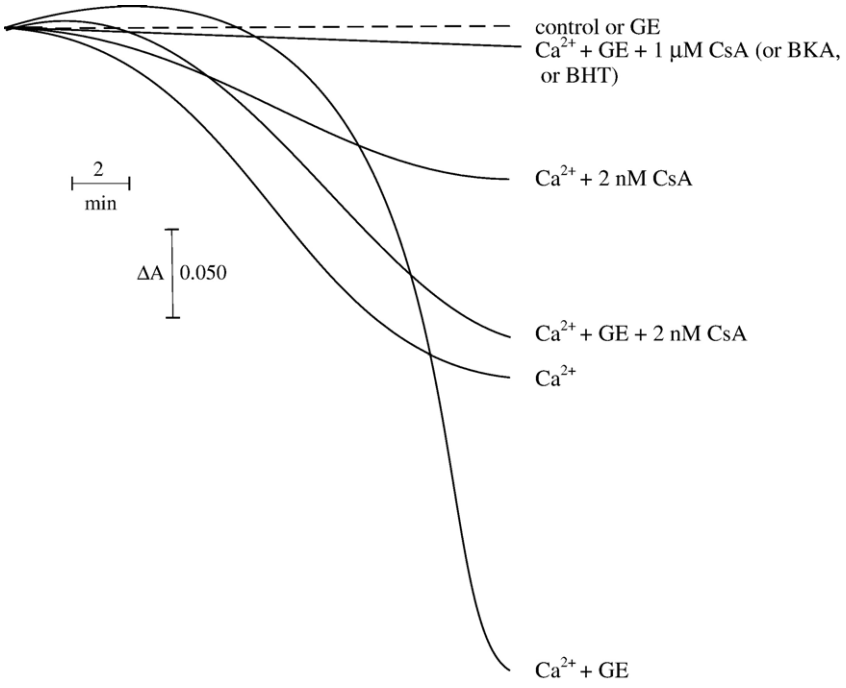


Fig. 2. Effect of various MPT inhibitors on swelling of RHM induced by Ca^{2+} or Ca^{2+} plus GE. RHM were incubated in standard medium, in conditions indicated in Materials and methods, with 200 μM Ca^{2+}. GE was present at concentration of 10 μM. CsA concentrations are also shown. Where indicated, 5 μM BKA or 50 μM BHT was present. Four other experiments gave overlapping identical curves.

exhibit any change. Treatment with 10 μM GE, in the above conditions, did not induce any osmotic change to the organelles either (Fig. 1). However, when RHM were incubated with 200 μM Ca^{2+}, the absorbance of the suspension decreased by about 0.2 units after 20 min of incubation, indicating low-amplitude swelling (Fig. 1). In these conditions, the presence of GE, again at 10 μM concentration, initially inhibited this process, but after 7–8 min of incubation had a reverse effect, inducing large-amplitude swelling.

At 5 μM, GE maintained complete inhibition of swelling throughout the experiment. GE at 1 or 2 μM, although to a lesser extent, also had an inhibitory effect. Instead, 7.5 μM GE did not affect the low-amplitude swelling induced by Ca^{2+}, but only showed slight initial protection. It should be noted that, in the presence of phosphate (Pi) (with Ca^{2+} concentrations lower than 200 μM in order to obtain the same absorbance decrease of 0.2 units), the inhibition by 5 μM GE was reduced or abolished, depending on Pi concentration (results not reported). The inset of Fig. 1 shows the dose-dependent effect of GE. It should be noted that higher concentrations of GE (e.g., 50 μM) only slightly enhanced the extent of MPT (results not reported). The presence of the immunosuppressant cyclosporin A (CsA) (1 μM), the adenine nucleotide translocase (AdNT) inhibitor, bongkrekic acid (BKA) or the antioxidant butylhydroxytoluene (BHT) completely inhibited the swelling induced by 200 μM Ca^{2+} alone, and also by Ca^{2+} plus 10 μM GE (Fig. 2). Accurate titrations of MPT inhibition by nanomolar CsA concentrations, in the absence or presence of 10 μM GE, did not show any difference in the percentage of inhibition, suggesting that GE does not act on the same target as CsA. In this regard, Fig. 2 shows only the effects of 2 nM CsA, which reached 50% of inhibition in both conditions.

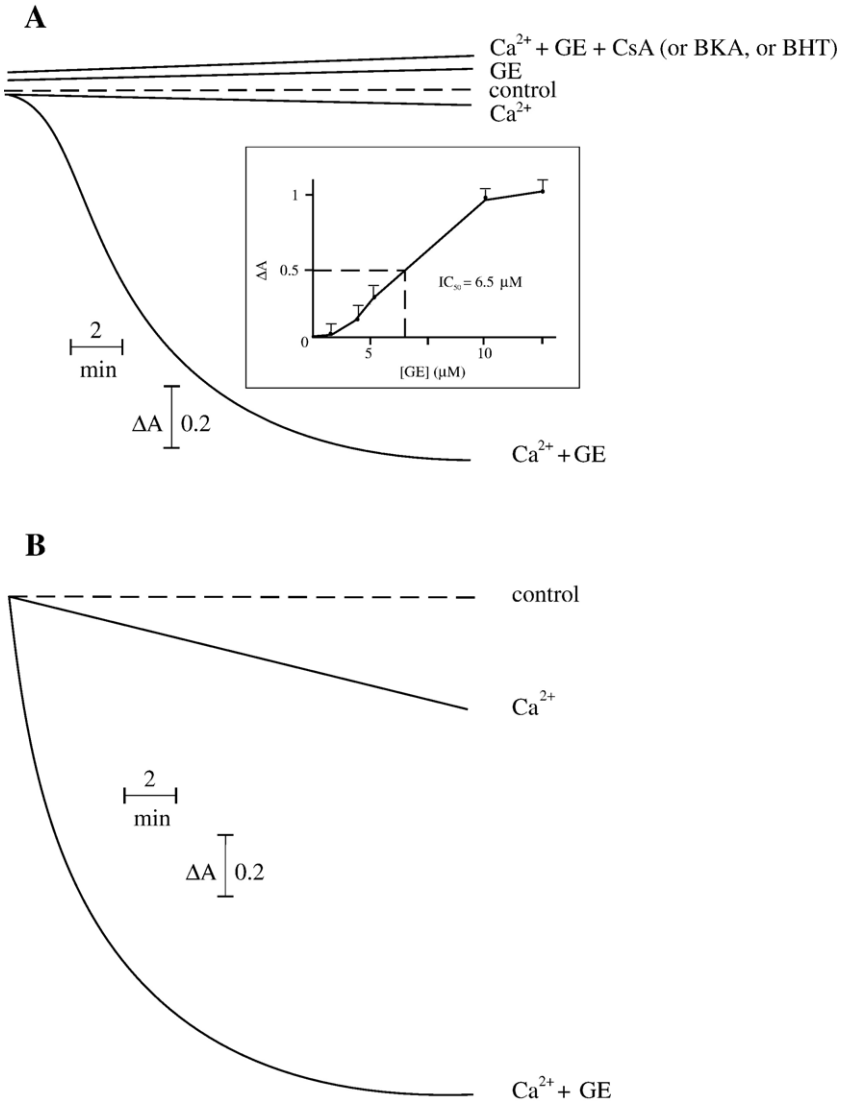


Fig. 3. Induction (A) or amplification (B) of mitochondrial swelling induced by GE in RLM in presence of Ca^{2+}. Effect of various MPT inhibitors. RLM were incubated in standard medium supplemented with 50 μM (A) or 200 μM (B) Ca^{2+}, in conditions indicated in Materials and methods. GE was present at a concentration of 10 μM. Where indicated, 1 μM CsA, 5 μM BKA or 50 μM BHT was present. Control curves (dashed lines) are in absence of Ca^{2+}. Downward deflection indicates mitochondrial swelling. Inset (A) shows dose-dependent effect of GE (with Ca^{2+}) calculated as in Fig. 1. Calculation of IC_{50} value for GE was evaluated as percentage of ΔA extent after 20 min, with respect to control. Four additional assays, for both panels A and B, exhibited same trend.

Significantly, as previously reported [8], in RLM GE behaves only as an inducer or amplifier of MPT. In fact, as shown in Fig. 3A (this figure is presented for better comparison with the new results in RHM), incubation of RLM in standard medium, in the presence of 50 μM Ca^{2+}, exhibited a very small change in the apparent absorbance of their suspension. However, when GE was added at 10 μM concentration, a strong decrease in absorbance was observed ($\Delta A \approx 1.0$), indicating the occurrence of considerable mitochondrial swelling (Fig. 3A). Instead, lower Ca^{2+} concentrations did not induce any change in absorbance, but 10 μM GE in the presence of the cation was still able to induce consistent swelling (results not reported). This effect was dose-dependent, with a $S_{0.5}$ value of 6.5 μM (see inset). The addition of CsA, BKA or BHT prevented this phenomenon (Fig. 3A). As shown in Fig. 3B, which reports new results obtained in liver, when RLM are treated with 200 μM Ca^{2+} (Fig. 3), they underwent considerable swelling, which was greatly accelerated and amplified in the presence of 10 μM GE. This swelling is also more rapid and more extensive than that observed in Fig. 3A.

The effect of GE in RLM has been ascribed to its pro-oxidant activity, that is, the capacity to induce oxidative stress resulting in mitochondrial pore opening [9]. Fig. 4A shows that, in RHM, both 200 μM Ca^{2+} and 10 μM GE, incubated separately over a period of 20 min, induced almost the same oxidation as thiol groups (about 20%). In these conditions, it should be noted that 200 μM Ca^{2+} can open the pore, although to a reduced extent, whereas 10 μM GE is ineffective (Fig. 1). When Ca^{2+} and GE were incubated together, thiol oxidation rose to over 30%, whereas BHT completely prevented it. When GE alone was present at 5 μM, no oxidation of thiols was observed. In addition, at this concentration, GE strongly prevented the oxidation induced by Ca^{2+} alone (Fig. 4A). The time-course of the effect of 5 and 10 μM GE on thiol oxidation in the presence or absence of Ca^{2+} is shown in Fig. 4B. In particular, it should be noted that the effect of 10 μM GE with Ca^{2+} was parallel to the time-course of swelling induction shown in Fig. 1. The inset in Fig. 4B shows the dose-dependent effect of GE on the redox level of thiols. GE initially strongly inhibited the gradual thiol oxidation induced by Ca^{2+}, but later failed to maintain it and induced further oxidation of thiols. Similar observations were obtained on the redox level of glutathione (Fig. 5). In fact, 200 μM Ca^{2+} and 10 μM GE, alone, induced oxidation of about 25%. When these agents were incubated together, glutathione

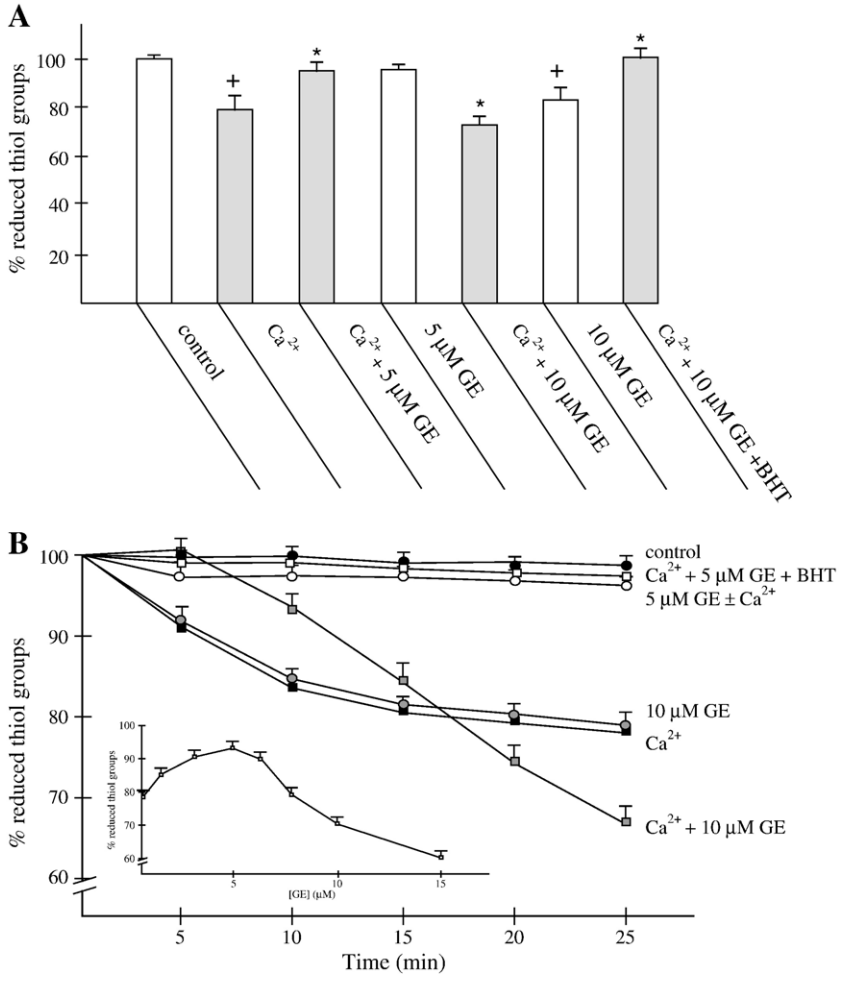


Fig. 4. Redox level of sulfydryl groups in presence of GE and Ca^{2+} (A). Time-dependent changes of redox level (B). Effect of BHT. RHM were incubated for 20 min in standard medium, in conditions indicated in Materials and methods, with or without 200 μM Ca^{2+}. GE was present at 5 or 10 μM concentration, as indicated. BHT was present at 50 μM concentration. Total content of reduced sulfydryl groups, considered as 100%: 92 < 0.05 vs. control, *p < 0.05 vs. Ca^{2+}).

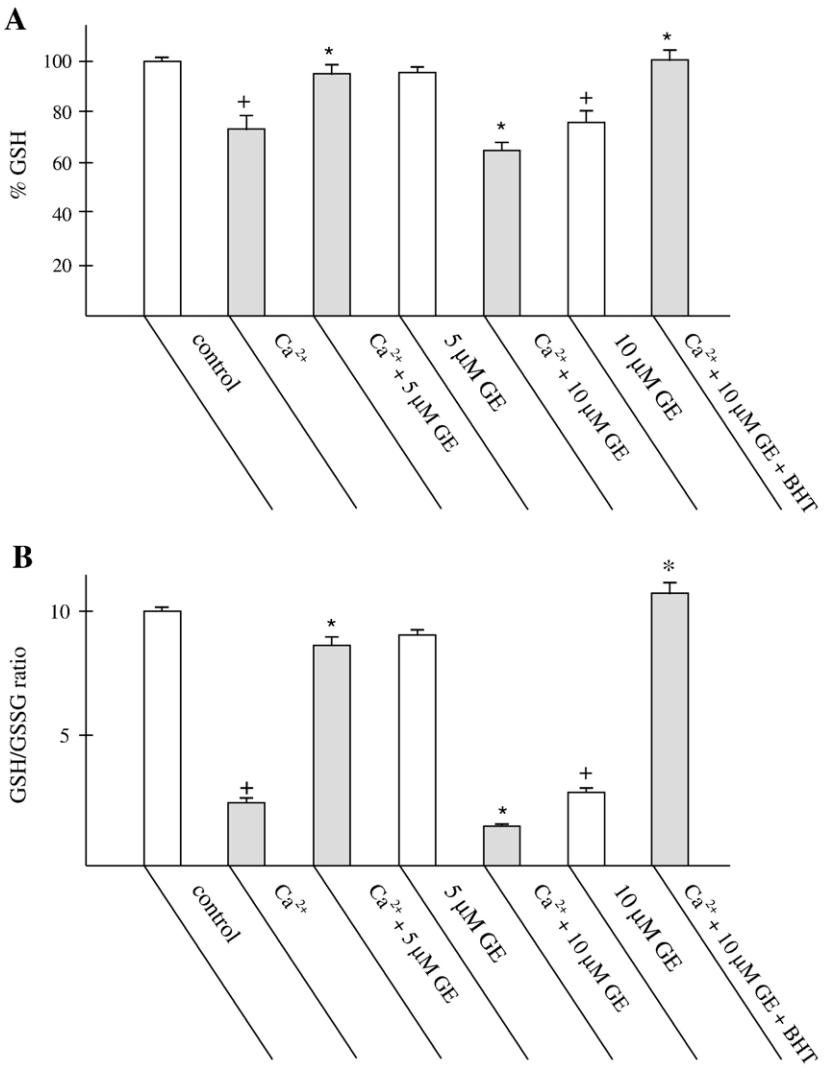


Fig. 5. Reduced glutathione change in presence of GE and Ca^{2+}. Effect of BHT. Incubation conditions, reagent concentrations and statistical analyses as in Fig. 4.

oxidation reached 35%, whereas BHT completely inhibited it. 5μM GE, alone, did not induce any oxidation and also prevented that induced by Ca^{2+}. The time-course of the effect of 10 μM GE plus Ca^{2+} on glutathione oxidation is almost identical to that of thiols shown in Fig. 4B (results not reported). The histogram of Fig. 5B shows the calculation of the GSH/GSSG ratio for the same conditions as in Fig. 5A. Results show that Ca^{2+} and 10 μM GE, when incubated alone, induce a considerable decrease of the ratio, when compared with the control. The ratio further decreases when the agents are incubated together, whereas BHT maintains it at the highest level. It should be noted that 5 μM GE, which alone shows a sufficiently high value of the ratio, can also preserve it in the presence of Ca^{2+}. In addition, GE concentrations higher than 10 μM (e.g., 25–50 μM) give rise to an almost negligible enhancement of oxidative stress (results not reported).

To identify the mechanism of this oxidative stress, RHM were treated with 10 μM GE plus 200 μM Ca^{2+}, in particular conditions capable of inducing different redox states in the respiratory chain (for a complete description of the procedure, see Ref. [8]). The results (not reported here) are almost identical to those obtained in RLM [8], so that it is possible to state that GE interacts with the N-2 Fe–S centre of Complex I also in RHM. This interaction produces a GE-derived oxygen-centred radical which, by reacting with O_2, forms the superoxide anion O_2^- and subsequently hydrogen peroxide and hydroxyl radical [8]. The latter species is most probably responsible for the oxidative stress.

These results prove that GE at a higher concentration (10 μM) exhibits a dual time-dependent effect on Ca^{2+}-induced pore opening. Initially it behaves as an inhibitor, and then as an amplifier of the phenomenon. Lower concentrations of GE only result in inhibition of thiol oxidation. Complete or partial inhibition, depending on GE concentration, is most probably due to the amount of Ca^{2+} which enters the mitochondrial matrix in its presence. The results shown in Fig. 6 cover this assumption. Exogenous Ca^{2+} at 200 μM is rapidly accumulated by RHM for about 60 nmol/mg prot. and retained in the matrix for 12 min. Then the cation is released into the medium, due to the opening of the transition pore (see Fig. 1). This is demonstrated by the

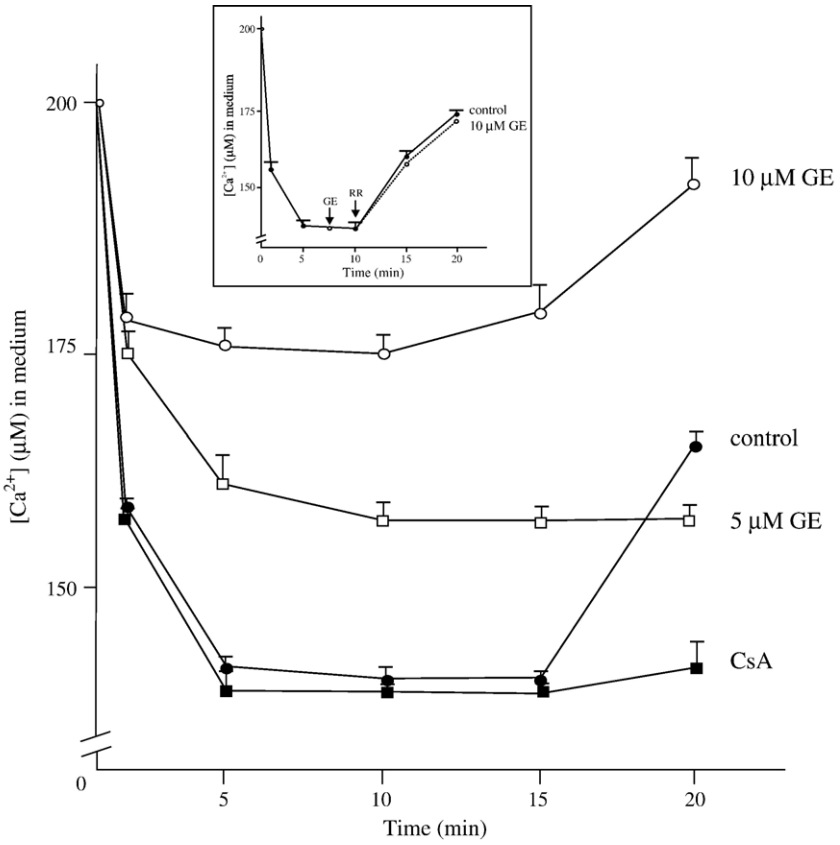


Fig. 6. Effect of GE on Ca^{2+} transport at various concentrations. RHM were incubated in standard medium, in conditions indicated in Materials and methods, with 200 μM Ca^{2+}. GE was present at 5 or 10 μM concentration, as indicated. CsA was present at 1 μM concentration. Ca^{2+} endogenous content was 15 nmol/mg prot. Inset: Ca^{2+} efflux induced by 2 μM ruthenium red (RR). Mean values ± SD of four experiments performed with atomic spectroscopy. Experiments with a Ca^{2+} selective electrode gave almost identical results (not reported).

effect of CsA, which retains Ca^{2+} inside the mitochondrial matrix by keeping the pore closed. The presence of GE at 5 and 10 μM reduces the amount of accumulated Ca^{2+} to about 40 and 25 nmol/mg prot., respectively. These Ca^{2+} levels in the mitochondrial matrix are responsible for the observed effects of GE on the MPT. In fact, at exogenous concentrations of Ca^{2+} which allows the cation to accumulate at 40 and 25 nmol/mg prot., MPT is not induced (results not reported). Accumulation of 25 nmol/mg prot. induces pore opening only in the presence of 10 μM GE (Figs. 1 and 6). It should be emphasised that Ca^{2+} efflux through the electroneutral transporter, induced by the addition of ruthenium red after Ca^{2+} accumulation, does not exhibit any variation in the absence or presence of GE added just before ruthenium red (Fig. 6, inset).

The release of the pro-apoptotic factors cytochrome *c* and AIF, after induction of mitochondrial swelling, which causes rupture of the external membrane, demonstrates that 200 μM Ca^{2+} induces the MPT and that considerable quantities of cytochrome *c* and AIF are released into the incubation medium (Fig. 7A), as also observed in RLM [8]. Fig. 7A also shows that 10 μM GE enhances the release of these factors, whereas 5 μM GE inhibits it, due to Ca^{2+} alone. CsA did completely prevent cytochrome *c* and AIF efflux, thus proving that it is the effect of pore opening. The release in the supernatant of cytochrome *c* by Ca^{2+}, in the absence or presence of 10 μM GE, is time-dependent and unequivocally confirmed by the remaining cytochrome *c* measured in mitochondrial pellets (Fig. 7B). The inhibition by 5 μM GE on Ca^{2+}-induced cytochrome *c* efflux, observed at different times, is also confirmed by the determination of cytochrome *c* in pellets (Fig. 7B). Time-dependent AIF release exhibits an identical trend (results not reported). The effect of 10 μM GE is comparable to the results on mitochondrial swelling, with a double action (see Fig. 1), i.e., inhibition after 5 min and an increase after 20 min. 10 μM GE, in the absence of Ca^{2+}, is ineffective (results not reported).

Cytochrome *c* and AIF release by 10 μM GE are accompanied by caspase activation, revealed by the cleavage of PARP (Fig. 8B) and apoptosis induction (Fig. 8A) in cardiomyocytes. It should be emphasised that the induction of apoptosis by GE has also recently been reported by other authors [17].

4. Discussion

The myocardium has a very high mitochondrial density and performs almost exclusively aerobic metabolism. This fact contributes to the greater production of ROS and a clearcut mechanism of detoxification of these compounds in comparison with other tissues [27]. We found the significantly different effect of GE in cardiac and hepatic mitochondria at high levels

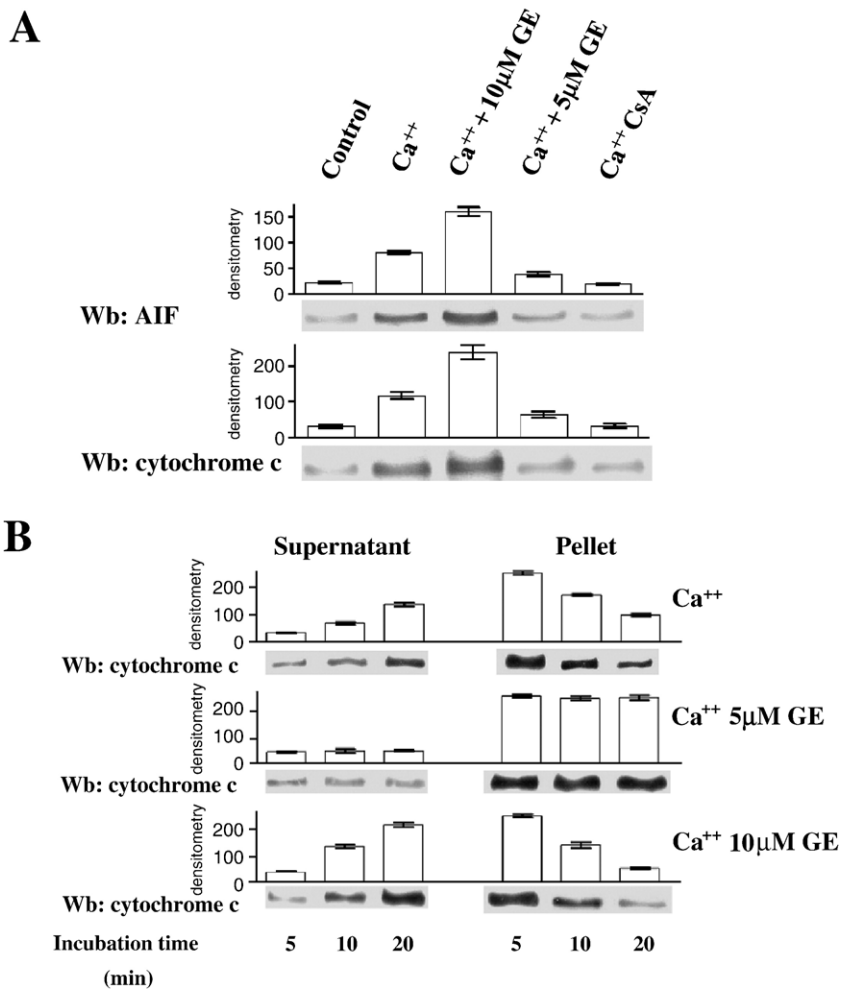


Fig. 7. Release of cytochrome *c* and AIF induced by Ca^{2+} and effect of GE at various concentrations (A). Time-dependent release of cytochrome *c* detected in supernatant and pellet (B). RHM were incubated in standard medium, in conditions indicated in Materials and methods, with 200 μM Ca^{2+}, for 20 min in panel A experiments and at indicated time in panel B. GE was present at concentrations indicated. CsA was at 1 μM concentration. Data are typical of three separate experiments.

of Ca^{2+}. The concentrations of Ca^{2+} used in this study, necessary to induce MPT, were much higher than physiological ones (around 0.1 μM). However, we used these levels of Ca^{2+}, which also occur in pathological conditions, to induce MPT by the cation alone, without other inducers, in order to detect only the effects of GE on this phenomenon. In this case, mitochondria, by means of their Ca^{2+} uniporter, behave as Ca^{2+} stores and participate in the regulation of Ca^{2+} concentrations in cytosol. It should be noted that, in cell cultures, MPT may be induced by very high-Ca^{2+} microdomains generated at the tight endoplasmic reticulum (ER)-mitochondrial junctions upon inositol triphosphate-mediated release of Ca^{2+} from ER [28].

As previously demonstrated in liver, at supraphysiological concentrations of Ca^{2+}, higher than 1 μM, GE induces the phenomenon of MPT [8] (see also Fig. 3). In the heart, besides the different response to Ca^{2+} (200 μM Ca^{2+} induces a ΔA decrease of 0.2 in RHM and 0.6 in RLM: compare Figs. 1 and 3B), two opposite effects take place, according to GE concentration. Mitochondrial swelling of low extent, caused by 200 μM Ca^{2+}, is completely inhibited by 5 μM GE (Fig. 1). This inhibition can also be achieved, although with reduced efficacy, at lower GE concentrations (1 or 2 μM). At 10 μM GE, the inhibition of swelling is also observed in the first 7–8 min of incubation. Afterwards, this protection completely fails and large-amplitude swelling occurs (Fig. 1). At 7.5 μM, GE is ineffective as either inhibitor or amplifier: most probably this is a threshold concentration, lower concentrations are inhibitors, and higher ones are amplifiers (see Fig. 1, inset). Worthy of note is the fact that these GE concentrations are very close to that of the plasma of free GE after licorice ingestion [29], and were in fact used in a recent study on isolated RHM and cardiomyocytes [17].

The low-amplitude swelling induced by Ca^{2+} and the amplification induced by GE are both the result of pore opening, although to different extents, as they are prevented by the typical inhibitors of MPT, CsA and BKA, and by the ROS scavenger BHT, as shown in Fig. 2. The effects of GE in protecting or amplifying MPT are paralleled by the retention or release of cytochrome *c* and AIF. In fact, 5 μM GE prevents the

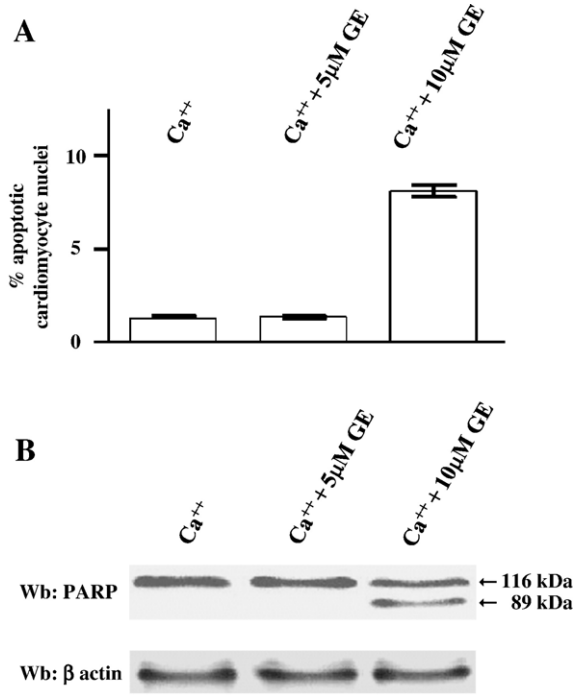
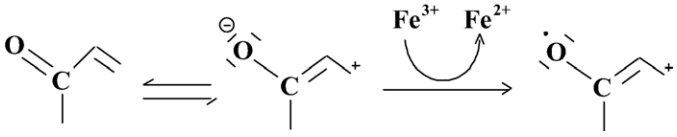


Fig. 8. Apoptotic death in rat cardiomyocyte cultures induced by GE. Percentage of apoptotic nuclei (A). Cleavage of PARP (B). Cardiomyocytes were cultured for 24 h in DMEM, in absence (control) or presence of 5 and 10 μM GE, as indicated. (A) Percentage of apoptotic nuclei was scored as per cent of total number analysed (1000). Each value represents mean ± SD of four experiments. (B) Caspase activity was analysed by Western blotting with antibody against PARP protein, a caspase substrate. Four other experiments gave almost identical results. Blot is shown with anti-β-actin antibody as loading control.

release of the pro-apoptotic factors induced by Ca^{2+}, whereas 10 μM GE further increases it (Fig. 7A, B), confirming the role of GE in modulating the triggering of the pro-apoptotic pathway in the heart. In this regard, induction of caspase activation and apoptosis by 10 μM GE has been demonstrated in rat cardiomyocytes (Fig. 8A, B).

We need to reassess our knowledge of the mechanism of pore opening mediated by Ca^{2+}, in order to explain the mechanism of action of GE in RHM. It is generally accepted that the occurrence of this event requires the interaction of Ca^{2+} with cardiolipins of the anular domain of AdNT. This would favour the interaction of this protein with cyclophilin D, which predisposes the opening of the transition pore [30]. However, the oxidation of two critical cysteines, located on AdNT, is also required for pore opening [31]. In conclusion, besides high concentrations of Ca^{2+}, the onset of oxidative stress, as evidenced by thiol and glutathione oxidation, and a strong decrease in the GSH/GSSG ratio (Figs. 4 and 5) are also necessary. This is also confirmed by the protective effect exhibited by BHT on swelling (Figs. 2 and 3A), thiol oxidation (Fig. 4A, B), glutathione oxidation (Fig. 5A) and the GSH/GSSG ratio (Fig. 5B). Calcium ions alone, at high concentrations, can also open the pore by inducing oxidative stress, as observed in the experiments reported here (Fig. 1). This effect is due to altered electron flux, consequent upon interaction with membrane cardiolipins which induces membrane disarrangement. This in turn leads to an accumulation of semiquinone radical which, by reacting with molecular oxygen, produces the superoxide anion O_2^- [32]. MPT induced by Ca^{2+} alone is generally of reduced extent (see Fig. 1), most probably because the production of ROS takes place far from the pore-forming structures and most of them react with other targets before reaching the critical cysteines. Induction of large-scale pore opening requires the action of a pro-oxidant, which produces ROS by interacting with the respiratory chain near the pore structures. This is the case of GE, as demonstrated in RLM [9] which, at a concentration of 10 μM, can generate ROS by reacting with the Fe–S centre, N-2, of Complex I of the respiratory chain The reactive site of GE is the conjugated carbonyl oxygen at C-11 (see molecular structure of GE in Ref. [9]), which may be involved in keto-enol tautomerism. This group interacts with the Fe^{3+} of the above-mentioned centre by producing a GE-derived oxygen-centred radical by means of the following reaction, also proposed by other authors [33]:



Thus, the centred oxygen radical reacts with O_2 to form the superoxide anion $O_2^{\cdot -}$ which in turn generates hydrogen peroxide. Then H_2O_2, by interacting with Fe^{2+}, produces the hydroxyl radical OH. by means of the Fenton reaction.

Lastly, in the presence of 50 μM Ca^{2+}, the hydroxyl radical induces oxidative stress, leading to the oxidation of the above-mentioned cysteines and the consequent induction of MPT (Fig. 2A). This concentration of Ca^{2+} alone does not have any oxidative and osmotic effect on RHM. In these mitochondria, at 5 μM concentration, GE prevents the low-amplitude swelling induced by 200 μM Ca^{2+} (Fig. 1). This effect is due to the protection of GE on thiol and glutathione oxidation by Ca^{2+} (Figs. 4 and 5), suggesting that the critical cysteine also remains reduced. This protection is the result of inhibited Ca^{2+} transport due to 5 μM GE (Fig. 6), but it is also due to the lack of thiol and glutathione oxidation by GE at this concentration (Figs. 4 and 5). It should be emphasised that the inhibition of Ca^{2+} uptake is the result of the inhibition of the Ca^{2+} uniporter, as Ca^{2+}/Na^+ exchange, responsible for Ca^{2+} efflux, is not affected (Fig. 6, inset). Confirmation of this mechanism of GE protection is given by the effect of Pi which, by collapsing ΔpH, increases $\Delta\Psi$, favouring Ca^{2+} transport and consequently reducing inhibition by GE (manuscript in preparation). One explanation is that the target of GE, the Fe–S centre N-2, in heart mitochondria, is located in a site which is more difficult to reach by GE than in liver mitochondria. At 10 μM concentration, GE also protects RHM against the MPT induced by 200 μM Ca^{2+} for several minutes. The mechanism for this protection is the same as that proposed above — that is, protection (for 7–8 min) against thiol oxidation (Fig. 4B) and strong inhibition of Ca^{2+} transport (Fig. 6). However, after this period of time, GE has the opposite effect, considering MPT and thiol oxidation. The pore rapidly opens to a large extent, and thiols and glutathione are oxidised more than in the presence of Ca^{2+} alone (Figs. 2, 4 and 5). In this regard, it should be emphasised that the dose-dependent effect of

GE on MPT (Fig. 1, inset) is closely related to its effect on the redox level of thiols (Fig. 4B, inset). The explanation is that, at first, GE cannot reach its target and inhibits Ca^{2+} transport. Then, when it reaches the Fe–S centre, ROS are produced, an important mitochondrial antioxidant defence system is impaired (decreased GSH level (Fig. 5A) and the GSH/GSSG ratio (Fig. 5B)), critical cysteines are oxidised, and MPT is induced. The observation that 10 μM GE strongly inhibits Ca^{2+} transport without preventing MPT induction means that the amount of cation transported, 25 nmol/mg prot., although unable to oxidise the critical thiol groups, is sufficient to predispose AdNT to open the pore. Inhibition by GE on mitochondrial Ca^{2+} transport explains the observed increase in cytosol Ca^{2+} levels induced by simulated ischaemia in cardiomyocytes in the presence of the drug [34]. The presence of various isoforms of the protein connexin in the mitochondria of heart (Cx43) and liver (Cx32) [17] may explain the differing effects of GE in both these organs. That is, the drug interacts with these isoforms by inhibiting the formation of gap junctions and, more in general, the mitochondrial bioenergetic function [17]. One proposal is that the cardiac isoform Cx43 interacts more strongly with GE than with the liver isoform Cx32. This allows the drug to overcome this hindrance and reach its target, but only at concentrations higher than 7.5 μM. Instead, the liver isoform Cx32, most probably by establishing a weaker interaction with GE, is able to reach the target even at concentrations lower than 7.5 μM.

In conclusion, these results clearly explain the mechanism of the induction of mitochondrial-mediated apoptosis by GE in cardiomyocytes, as recently observed [17]. They also emphasise two important differences between the structure of heart and that of the liver mitochondrial membrane: i) the target of GE in RHM is less accessible, most probably due to the presence of the isoform Cx43, ii) the Ca^{2+} uniporter in RHM is sensitive to GE.

There are few data suggesting that GE has different physiological effects in liver and heart. However, the results here reported suggest that further investigations should be initiated in this field.

The different characteristics of the membrane mentioned above confirm tissue specificity by GE with regard to RHM and RLM. From a physiological point of view, it has been reported that the pro-apoptotic effect of GE or its derivative, carbenoxolone, observed in liver, is due not only to the inhibition of 11HSD but also to MPT induction, thus indicating the toxic effect of GE at mitochondrial level [9]. In fact, in some tissues, this toxicity may have a beneficial effect at cellular level by inducing programmed cell death, which is considered a defence against neoplastic proliferation.

In the heart, the local effect of relatively low amounts of circulating GE can establish a protection system against programmed cell death. Also, in the heart, GE behaves as a gap junction uncoupler — that is, it can prevent the cell-to-cell progression of hypercontracture in cardiomyocytes and attenuated myocardial injury when given during reperfusion. This uncoupling protects the heart against cell death occurring after the following reoxygenation [34]. The observed protection against MPT by GE at 5 μM, and the inhibition of mitochondrial Ca^{2+} transport, can also prevent cell death in conditions of exogenous (cytosol) high Ca^{2+} concentrations. In addition, the protection against oxidative stress observed at lower concentrations fits the antioxidant activity demonstrated *in vivo* in rats treated with isadrine and the beneficial effects against cardiac disorders accompanied by inflammatory and necrotic changes in the myocardium [35]. The different metabolic pattern of liver and heart mitochondria is probably also affected *in vivo* by other factors, such as the availability of aldosterone, cortisol, mineralocorticoid receptors and 11HSD types 1 and 2 at heart level. When licorice is consumed in high amounts or aldosterone is produced in excess, the mitochondrial and non-mitochondrial production of oxidative stress due to the two substances may be synergic, interacting in the onset of heart failure. In other situations, e.g., during consumption of low amounts of licorice, the beneficial effect of this at the level of mitochondria may prevail, if GE plasma concentrations are still not able to block 11HSD type 2 and mineralocorticoid receptors.

Acknowledgement

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

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