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Glycyrrhizin and glycyrrhetinic acid directly modulate rat cardiac performance

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Abstract

Root extract of liquorice is traditionally used to treat several diseases. Liquorice-derived constituents present several biological actions. In particular, glycyrrhizin and its aglycone, glycyrrhetinic acid, exhibit well-known cardiovascular properties. The aim of this research was to explore the direct cardiac activity of glycyrrhizin and glycyrrhetinic acid.

The effects of synthetic glycyrrhizin and glycyrrhetinic acid were evaluated on the isolated and Langendorff perfused rat heart. The intracellular signaling involved in the effects of the two substances was analyzed on isolated and perfused heart and by Western blotting on cardiac extracts. Under basal conditions, both glycyrrhizin and glycyrrhetinic acid influenced cardiac contractility and relaxation. Glycyrrhizin induced significant positive inotropic and lusitropic effects starting from very low concentrations, while both inotropism and lusitropism were negatively affected by glycyrrhetinic acid. Both substances significantly increased heart rate. Analysis of the signal transduction mechanisms suggested that glycyrrhizin acts through the endothelin receptor type A/phospholipase C axis while glycyrrhetinic acid acts through endothelin receptor type B/Akt/nitric oxide synthase/nitric oxide axis.

To our knowledge, these data reveal, for the first time, that both glycyrrhizin and glycyrrhetinic acid directly affect cardiac performance. Additional information on the physiological significance of these substances and their cardiac molecular targets may provide indication on their biomedical application.
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Keywords: Glycyrrhizin; Glycyrrhetinic acid; Myocardial contractility; Endothelin-1; Nitric oxide

1. Introduction

Liquorice derives from root extract of *Glycyrrhiza glabra*, a perennial herb cultivated in temperate and subtropical regions. Due to its sweet taste, it is widely applied as a conditioning and flavouring agent in various consumption products. Since ancient times, liquorice roots were used in traditional herbal medicine for treatment of a large range many diseases [1,2]. Only in the last 25 years the effects of *Glycyrrhiza* compounds have been scientifically investigated, confirming the knowledge acquired during history [3].

Liquorice constituents exhibit several biological and endocrine properties including anti-inflammatory (cortisol-like), antihepatotoxic, antibacterial, antiviral and anticancer effects [4–7]. In addition, they possess cardioprotective properties [8,9].

Glycyrrhizin (GA), the main constituent of *G. glabra*, is a glycoside, which occurs as a mixture of calcium, sodium and potassium salts [6]. Orally administered, GA is poorly absorbed by the intestinal tract and hydrolyzed by β-D-glucuronidase-containing intestinal bacteria in two molecules of D-glucuronic acid and the aglycone glycyrrhetinic acid (GE), a pentacyclic triterpene [10]. If intravenously administered, GA is metabolized in the liver by lysosomal β-D-glucuronidase to 3-mono-glucuronide glycyrrhetinic acid. This metabolite is excreted with bile into the intestine, where it is transformed by bacteria into GE, which can be reabsorbed, causing a pronounced delay in the terminal plasma clearance.

Liquorice-induced hypertension is the well-known action exerted by both GA and GE. This effect results from the inhibition of 11β-hydroxysteroid dehydrogenase 2 (11β-HSD2), a key enzyme in cortisol metabolism. Hypertension induced by 11β-HSD2 inhibition may involve not only glucocorticoid and mineralocorticoid receptor-mediated modulation of the renal function but also the modulation of the cardiovascular endothelin (ET)-1 and nitric oxide (NO) systems [11].

In rabbit [9] and rat [8,12], GA and GE were shown to be cardioprotective. This cardioprotection involves different pathways: in rabbit, GA, but not GE, decreases neutrophil influx and myocardial infarct size after regional myocardial ischemia/reperfusion [9]; in rat cardiac mitochondria, GE amplifies mitochondrial permeability and concomitant release of proapoptotic factors [8]. In particular, GE, acting as gap junction inhibitor, influences connexin 43, the major gap-junction-forming protein in the adult cardiac ventricles and a regulator of mitochondrial function [8,13].

Although new aspects of liquorice pharmacology have been recently uncovered, the bulk of the studies referred to the in vivo effects of liquorice after oral administration. So far, the direct action

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on tissues and organs received very little attention, and nothing is known regarding the effects directly elicited by GA and GE on myocardial contractility and relaxation.

We investigated whether GA and GE affect the inotropic, lusitropic, chronotropic and coronary performance of the mammalian heart and the possible signal transduction pathways involved. For this purpose, we utilized the isolated and Langendorff perfused rat heart preparation, which is an ideal model for analyzing the direct cardiac effects of a substance without extrinsic neuronal and endocrine influences. The intracellular signaling involved in GA and GE effects was analyzed on both isolated and perfused hearts and by Western blotting on cardiac extracts.

2. Methods and materials

2.1. Animals

Male Wistar rats (Harlan Laboratories s.r.l., Udine, Italy) weighing 180–240 g were housed in a ventilated cage rack system under standard conditions. Animals had food and water access ad libitum. Animal care, sacrifice and experiments were supervised according to the *Guide for the Care and Use of Laboratory Animals* published by US National Institutes of Health (Publication No. 85-23, revised 1996).

2.2. Isolated Langendorff heart preparation

Rats were anesthetized with intraperitoneal injection of ethyl carbamate (2 g/kg rat, ip), and the hearts were rapidly excised and transferred in ice-cold buffered Krebs–Henseleit solution (KHs). As previously described [14], the aorta was immediately cannulated with a glass cannula and connected to the Langendorff apparatus to start perfusion at constant flow-rate (12 ml/min). Briefly, the apex of the left ventricle (LV) was pierced to avoid fluid accumulation. A water-filled latex balloon, connected to a BLPR gauge (BLPR, World Precision Instruments, Sarasota, FL, USA), was inserted through the mitral valve into the LV to allow isovolumic contractions and to continuously record mechanical parameters. Another pressure transducer located just above the aorta recorded coronary pressure (CP). The perfusion solution consisted of a modified nonrecirculating KHs containing (in mmol/L) NaCl 113, KCl 4.7, $NaHCO_3$ 25, $MgSO_4$ 1.2, $CaCl_2$ 1.8, KH_2PO_4 1.2, glucose 11, mannitol 1.1 and Na-pyruvate 5 (pH 7.4; 37°C; 95% O_2–5% CO_2). Haemodynamic parameters were assessed using a PowerLab data acquisition system and analyzed using a Chart software (both purchased from ADInstruments, Basile, Italy).

2.3. Basal conditions

The performance of the isolated and Langendorff perfused rat heart was evaluated for the inotropic effect by analyzing the left ventricular pressure (LVP, in mmHg), an index of contractile activity; the rate-pressure product (RPP, in mmHg beats/min), an index of cardiac work; the maximal value of the first derivative of LVP [+(LVdP/dt) max, in mmHg/s] which indicates the maximal rate of left ventricular contraction; the time to peak tension of isometric twitch (TTP, in s). For the lusitropic effect, the maximal rate of left ventricular pressure decline of LVP [−(LVdP/dt)max, in mmHg/s]; the half time relaxation (HTR, in s), which is the time required for tension to fall from the peak to 50%, and T/−t ratio obtained by +(LVdP/dt)max/−(LVdP/dt)max have been analyzed. Heart rate (HR, in beats/min) was measured for the chronotropic effect. Mean CP (mmHg) was calculated as the average of values obtained during several cardiac cycles [14].

2.3.1. GA and GE stimulated preparations

Preliminary experiments obtained by repetitive exposure of each heart to one concentration of either GA or GE (10^{-8} mol/L) revealed the absence of desensitisation (data not shown). Thus, concentration–response curves were generated by perfusing the cardiac preparations for 10 min with KHs with increasing concentrations (from 10^{-12} mol/L to 10^{-5} mol/L) of either GA or GE.

2.3.2. Involvement of ET receptors

To verify the involvement of ET receptors (ETRs) in the mechanism of action of GA and GE, experiments were performed by perfusing the hearts with KHs enriched either with GA (10^{-8} mol/L) plus a single concentration of BQ123 (10^{-7} mol/L), ETR_A selective inhibitor, or with GE (10^{-8} mol/L) plus a single concentration of BQ788 (10^{-7} mol/L), ETR_B selective inhibitor.

The antagonist concentration was selected on the bases of preliminary dose–response curves as the first dose which does not significantly affect the cardiac performance.

2.3.3. GA signal transduction mechanism

To obtain information about the involvement of the ETR_A/phospholipase C (PLC) system on GA-elicited cardiac and coronary actions, hearts were perfused with KHs enriched with a single concentration of GA (10^{-8} mol/L) alone and of GA (10^{-8} mol/L) plus a single concentration of U73122 (10^{-5} mol/L), a specific PLC inhibitor.

2.3.4. GE signal transduction mechanism

To obtain information about the involvement of ETR_B/NOS/NO system on GE-elicited cardiac and coronary actions, hearts were perfused with KHs enriched with a single concentration of GE (10^{-8} mol/L) alone and of GE (10^{-8} mol/L) plus a single concentration of either L-NAME (10^{-5} mol/L), a specific NOS inhibitor, or ODQ (10^{-5} mol/L), a specific sGC inhibitor, or KT5823 (10^{-7} mol/L), a specific PKG inhibitor.

2.3.5. Western blotting

Cardiac ventricles obtained after perfusion with a single concentration of GA (10^{-8} mol/L) or GE (10^{-8} mol/L) were homogenized in ice-cold RIPA buffer (Sigma-Aldrich, Milan, Italy) containing a mixture of protease inhibitors (1 mmol/L aprotinin, 20 mmol/L phenylmethylsulfonyl fluoride and 200 mmol/L sodium orthovanadate). Homogenates were then centrifuged at 200*g* for 10 min at 4°C to remove debris. Protein concentration was determined using Bradford reagent according to the manufacturer's recommendations (Sigma-Aldrich, Milan, Italy). Proteins were separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis gels, transferred to membrane (GE Healthcare, Milan, Italy), blocked with nonfat dried milk and incubated overnight at 4°C with anti-endothelial-NOS (eNOS) antibody (Sigma-Aldrich, Milan, Italy) or anti-phospho-eNOS antibody or anti-Akt antibody or anti-phospho-Akt antibody (Santa Cruz Biotechnology, DBA, Italy). Immunodetection was performed by using the Enhanced Chemiluminescence system (GE Healthcare, Milan, Italy).

2.4. Solutions and drugs

All chemicals/drugs were purchased from Sigma Chemical Co. (Sigma-Aldrich, Milan, Italy). ODQ was prepared in ethanol, while the other solutions were prepared in double-distilled water; dilutions were made in KHs solution immediately before use. KT5823 was used in a darkened perfusion apparatus to prevent degradation.

2.5. Statistical analysis

Data are expressed as the mean±S.E.M. Since each heart represents its own control, the statistical significance of differences within-group was assessed using the analysis of variance (ANOVA) test ($P<.05$). Comparison between groups was made by using a one-way ANOVA followed by the Bonferroni correction for post hoc *t* tests. Differences were considered to be statistically significant for $P<.05$.

3. Results

3.1. Basal conditions

After 20 min of stabilization, the following basal recordings were measured: LVP=89±3 mmHg, RPP=2.5±0.1×10^4 mmHg beats/min, +(LVdP/dT)max=2492±129 mmHg/s, TTP=0.08±0.01 s, −(LVdP/dT)max=1663±70 mmHg/s, HTR=0.05±0.01 s, T/−t or +(LVdP/dT) max/−(LVdP/dT)max=1.49±1.84 mmHg/s, HR=280±7 beats/min, CP=63±3 mmHg. Endurance and stability of the preparation, analyzed by measuring performance variables every 10 min, showed that the heart preparation is stable for up to 180 min.

3.2. GA inotropic, lusitropic, chronotropic and coronary actions

To verify whether GA affects basal cardiac performance, hearts were exposed to increasing GA concentrations (10^{-12} mol/L to 10^{-5} mol/L) to generate concentration–response curves. The effects of GA remained stable until 15–20 min. Thus, cardiac parameters were measured at 10 min.

Glycyrrhizin caused concentration-dependent positive inotropic (Fig. 1A) and lusitropic (Fig. 1B) effects, shown by a highly significant increase of LVP, +(LVdP/dt)max and −(LVdP/dt)max at all concentrations tested (10^{-12} mol/L to 10^{-5} mol/L). Moreover, all GA concentrations significantly increased HR (Fig. 1C) and CP (Fig. 1D). Glycyrrhizin effects persist in electrically paced preparations (data not shown), indicating their independence from chronotropism.

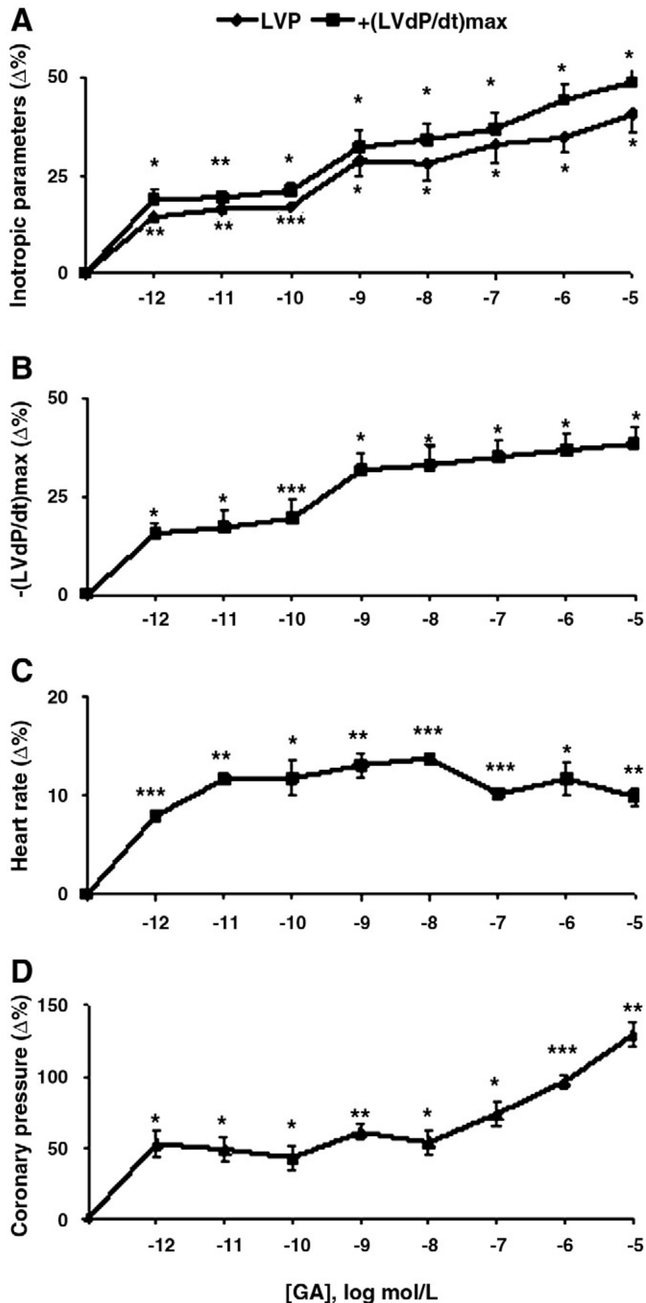

Fig. 1. Isolated Langendorff heart. Concentration-dependent response curves of GA (from 10^{-12} to 10^{-5} mol/L) on LVP and +(LVdP/dt)max (A), −(LVdP/dt)max (B), HR (C), CP (D). For abbreviations and basal values, see Results. Percentage changes were evaluated as means±S.E.M. of 7 experiments. Significance of difference from control values was done by one-way ANOVA: *$P<.05$; **$P<.01$; ***$P<.001$.

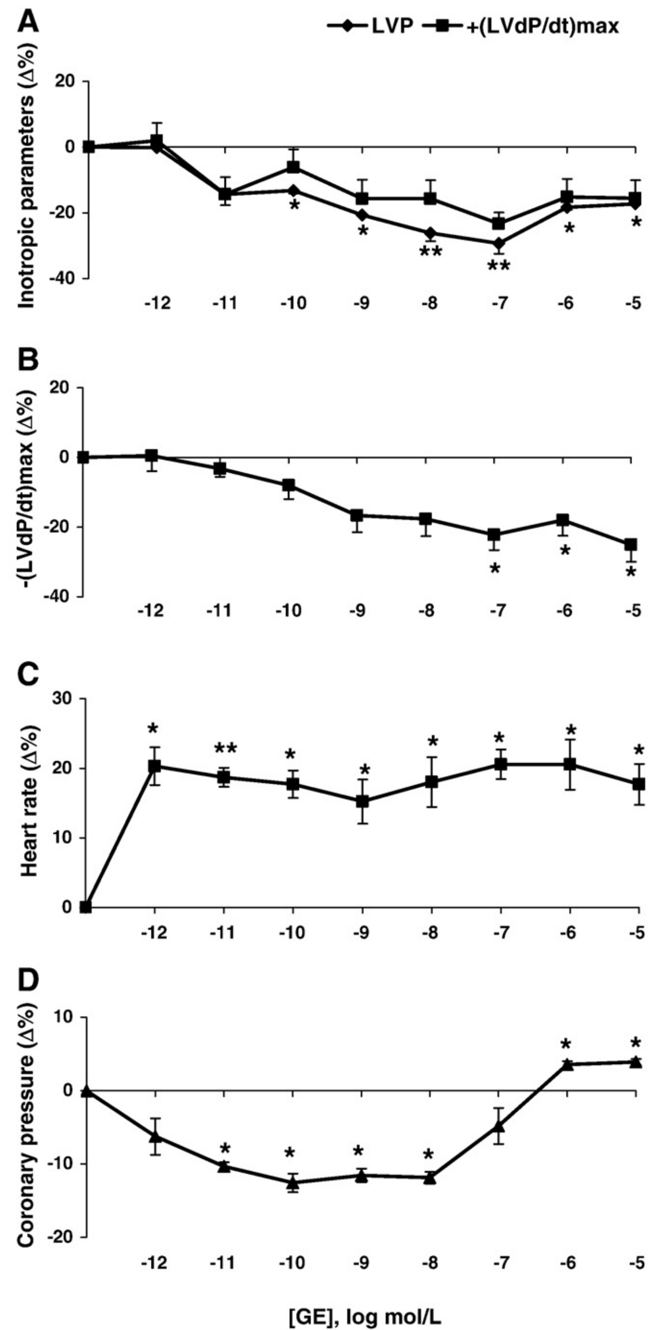

Fig. 2. Isolated Langendorff heart. Concentration-dependent response curves of GE (from 10^{-12} to 10^{-5} mol/L) on LVP and +(LVdP/dt)max (A), −(LVdP/dt)max (B), HR (C), CP (D). For abbreviations and basal values, see Results. Percentage changes were evaluated as means±S.E.M. of 7 experiments. Significance of difference from control values was done by one-way ANOVA: *$P<.05$; **$P<.01$.

3.3. GE inotropic, lusitropic, chronotropic and coronary actions

To verify whether GE affects basal cardiac performance, hearts were exposed to increasing GE concentrations (10^{-12} mol/L to 10^{-5} mol/L) to generate concentration–response curves. The effects of GE remained stable until 15–20 min. Thus, cardiac parameters were measured at 10 min.

Glycyrrhetinic acid caused concentration-dependent negative inotropic (Fig. 2A) and lusitropic (Fig. 2B) effects, shown by a decrease of LVP, +(LVdP/dt)max and −(LVdP/dt)max, significant starting from 10^{-10} mol/L (inotropic effect) or 10^{-9} mol/L (lusitropic effect). Moreover, GE induced an increase of HR (Fig. 2C), significant from 10^{-12} mol/L to 10^{-5} mol/L, and a concentration-dependent biphasic effect on CP, shown by a marked vasodilatation from 10^{-12} mol/L to 10^{-7} mol/L and a modest, but significant, vasoconstriction at 10^{-6} mol/L and 10^{-5} mol/L (Fig. 2D). Glycyrrhetinic acid effects persisted in electrically paced preparations (data not shown), indicating their independence from chronotropism.

3.4. GA signal transduction mechanism

It is known that GA acts through ET-1/ETR_A system [11]. To analyze the involvement of ETR_A in GA cardiac effects, experiments

were performed by perfusing the hearts with KHs enriched with a single concentration of GA (10^{-8} mol/L) alone and in the presence of BQ123 (10^{-7} mol/L), a selective ETR_A inhibitor. As shown in Fig. 3A, ETR_A blockade with BQ123 abolished all GA cardiac effects. To analyze ETR_A-dependent signal transduction, we tested a single concentration of GA (10^{-8} mol/L) alone and in the presence of U73122 (10^{-5} mol/L), a specific PLC inhibitor. Pretreatment with U73122 reversed both GA-dependent inotropic and lusitropic effects and abolished chronotropic effects, while increased GA-induced vasoconstriction (Fig. 3B).

3.5. GE signal transduction mechanism

It is known that the ETR_B may be involved in vasodilatation and negative inotropic effects [15]. To analyze the involvement of ETR_B in the cardiac effects of GE, experiments were performed by perfusing the hearts with KHs enriched with a single concentration of GE (10^{-8} mol/L) alone and in the presence of BQ788 (10^{-7} mol/L), a selective ETR_B inhibitor. As shown in Fig. 4, ETR_B blockade with BQ788 abolished all cardiac effects of GE. Since the interaction between ETR_B activation and the NOS/NO system is well known, we tested the effects of GE in the presence of inhibitors of NOS (L-NAME 10^{-5} mol/L), sGC (ODQ 10^{-5} mol/L) and PKG (KT5823 10^{-7} mol/L). All inhibitors abolished inotropic, lusitropic and chronotropic effects induced by GE while reversing GE-dependent effect on vasomotility (Fig. 5A, B, C). Moreover, to evaluate whether Akt–eNOS cascade is

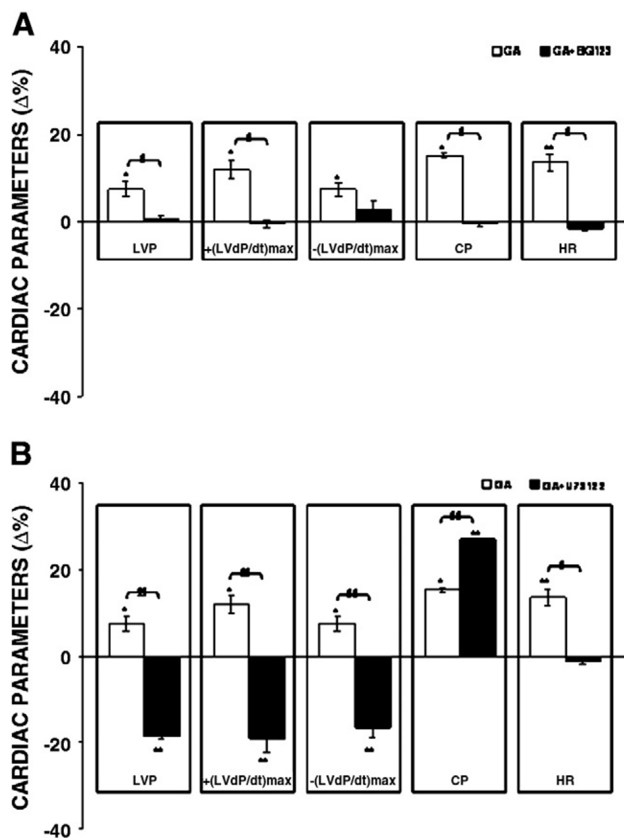

Fig. 3. Isolated Langendorff heart. Effects of GA (10^{-8} mol/L) plus BQ123 (10^{-7} mol/L) on LVP, +(LVdP/dt)max, −(LVdP/dt)max, CP and HR (A). GA (10^{-8} mol/L) plus U73122 (10^{-5} mol/L) on LVP, +(LVdP/dt)max, −(LVdP/dt)max, CP and HR (B). For abbreviations and basal values, see Results. Percentage changes were evaluated as means±S.E.M. of 7 experiments for each group. Significance of difference from control values was done by one-way ANOVA: *P<.05; **P<.01. Significance of difference from GA treatment was done by one-way ANOVA: §P<.05; §§P<.01.

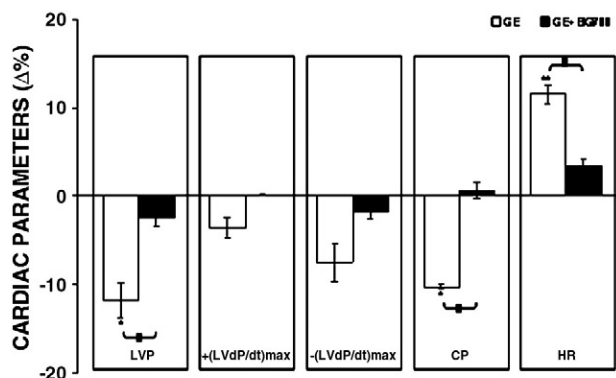

Fig. 4. Isolated Langendorff heart. Effects of GE (10^{-8} mol/L) plus BQ788 (10^{-7} mol/L) on LVP, +(LVdP/dt)max, −(LVdP/dt)max, CP and HR. For abbreviations and basal values, see Results. Percentage changes were evaluated as means±S.E.M. of 7 experiments for each group. Significance of difference from control values was done by one-way ANOVA: *P<.05; **P<.01. Significance of difference from GE treatment was done by one-way ANOVA: §P<.05.

recruited in the cardiovascular effects elicited by GE, the phosphorylation of these proteins was analyzed in cardiac extracts after exposure to GE (from 10^{-12} mol/L to 10^{-5} mol/L). We observed that GE elicits eNOS and Akt phosphorylation (Fig. 6A, B).

4. Discussion

4.1. GA and GE effects on myocardium and coronary reactivity

To our knowledge, the present study provides the first evidence that, under basal conditions, the major liquorice-derived products GA and its aglycone GE affect cardiac performance, showing dissimilar effects and mechanisms of action.

On the isolated and Langendorff perfused rat heart, used as a prototype of mammalian heart, both GA and GE influenced contractility and relaxation. In particular, GA induced significant positive inotropic and lusitropic effects starting from very low concentrations. In contrast, GE negatively affects inotropism and lusitropism from 10^{-10} mol/L to 10^{-5} mol/L. The effects of both GA and GE persist in electrically paced preparations (data not shown), indicating their independence from chronotropism. Also at the vascular level, these substances exerted different effects. While GA induced a notable and significant increase of CP at all concentrations tested, GE showed a significant vasodilation at low concentrations.

In both rat and humans, the bioavailability of GA after ingestion is low since it is rapidly hydrolyzed by commensal bacteria, thus forming its aglycone GE which is absorbed in a dose-dependent way. The pharmacokinetics of GA and GE in rats has been extensively described by Ploeger and coworkers [10 and references therein]. However, if intravenously administered, GA is present in plasma, before being metabolized in the liver [10]. Of note, the direct cardiac effects observed in our experiments after GA exposure are obtained starting from very low concentrations, which are comparable to those encountered in rat plasma after liquorice ingestion (~20 μg/ml) [10]. Accordingly, it is possible that, despite the very limited amounts, circulating GA reaches the heart, being able to potentiate its performance.

In contrast to GA, GE elicited negative inotropic and lusitropic effects and coronary dilation, suggesting that the molecular modifications occurring after hydrolysis of GA change its effects on cardiac activity. This is not surprising since the two substances were shown to be differently active in relation, for example, to the inhibition elicited on 11β-HSD2 (i.e., the enzyme responsible for converting cortisol to the inactive cortisone), with GE being up to 1000 times more potent

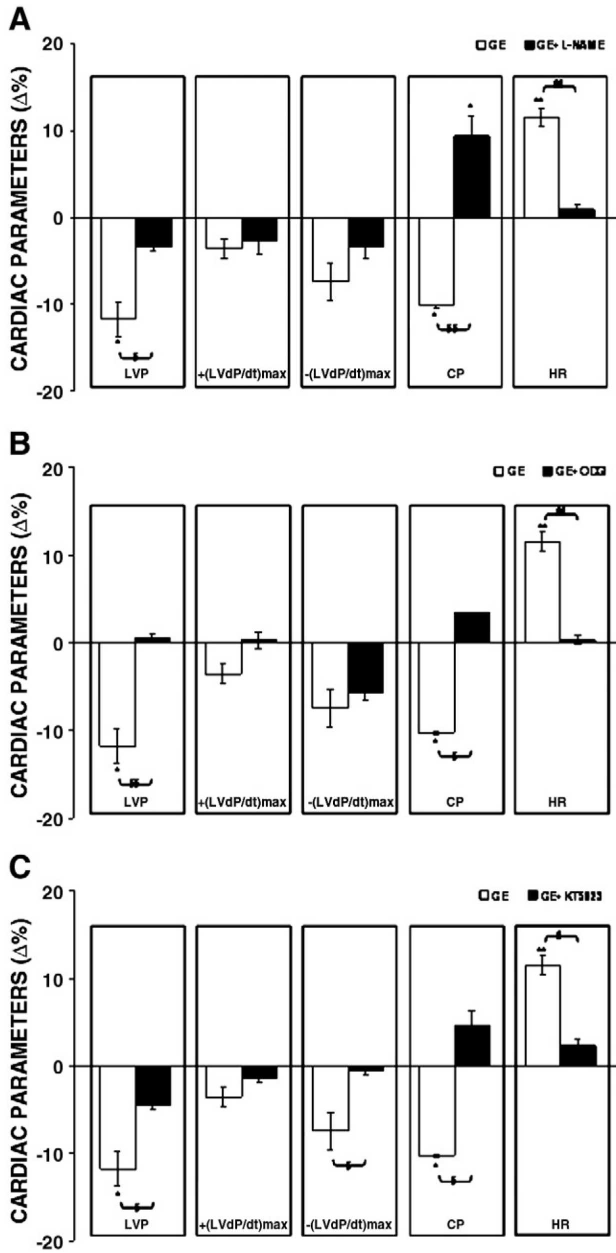

Fig. 5. Isolated Langendorff heart. Effects of GE (10^{-8} mol/L) plus L-NAME (10^{-5} mol/L) on LVP, +(LVdP/dt)max, −(LVdP/dt)max, CP and HR (A). GE (10^{-8} mol/L) plus ODQ (10^{-5} mol/L) on LVP, +(LVdP/dt)max, −(LVdP/dt)max, CP and HR (B). GE (10^{-8} mol/L) plus KT5823 (10^{-7} mol/L) on LVP, +(LVdP/dt)max, −LVdP/dt)max, CP and HR (C). For abbreviations and basal values, see Results. Percentage changes were evaluated as means±S.E.M. of 7 experiments for each group. Significance of difference from control values was done by one-way ANOVA: *P<.05; **P<.01. Significance of difference from GE treatment was done by one-way ANOVA: §P<.05; §§P<.01.

than GA [10]. The cardiodepression and the coronary dilation observed after GE exposure may deserve further investigations in order to establish whether this liquorice derivative may play a protective role in the presence of cardiac stress.

4.2. GA and GE signaling pathways

In our experiments, the analysis of the mechanism of action responsible for the cardiac responses elicited by GA and GE indicated an involvement of the ETRs system. In particular, GA-dependent effects are mediated by ETR_A activation, while GE-dependent effects are mediated by ETR_B. In fact, the vasoconstriction as well as the positive inotropism, lusitropism and chronotropism exerted by GA disappeared in the presence of specific ETR_A inhibition by BQ123. At the same time, the vasodilation, the positive chronotropism and the negative inotropism and lusitropism exerted by GE are abolished by specific ETR_B inhibition by BQ788. The involvement of the ETRs system in the liquorice-dependent effect has been already described. For example, the development of hypertension induced by the GA-dependent inhibition of 11β-HSD2 involves the vascular ET-1 system [11], although the role of the different ETRs subtypes has not been entirely clarified [16]. We report here that GA, through the ET-ETR_A pathway, positively affected inotropism and lusitropism via PLC stimulation with consequent hydrolysis of PIP_2 to DAG and IP_3, and increase of free calcium concentrations. In fact, application of the PLC

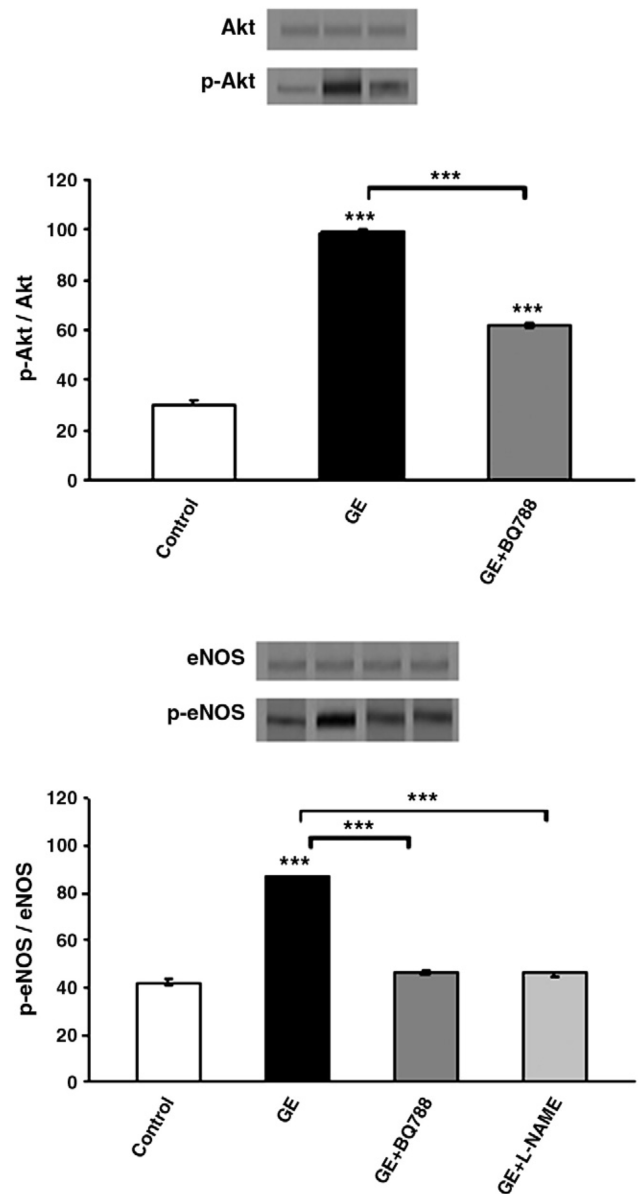

Fig. 6. Western blotting of p-Akt and total Akt in heart extracts and densitometric quantification of p-Akt over total Akt ratio (A). Western blotting of p-eNOS and total eNOS in heart extracts and densitometric quantification of p-eNOS over total eNOS ratio (B). Data are means±S.E.M. of 5 determinations for each group (n=3). Statistical differences were evaluated by one-way ANOVA: ***P<.001.

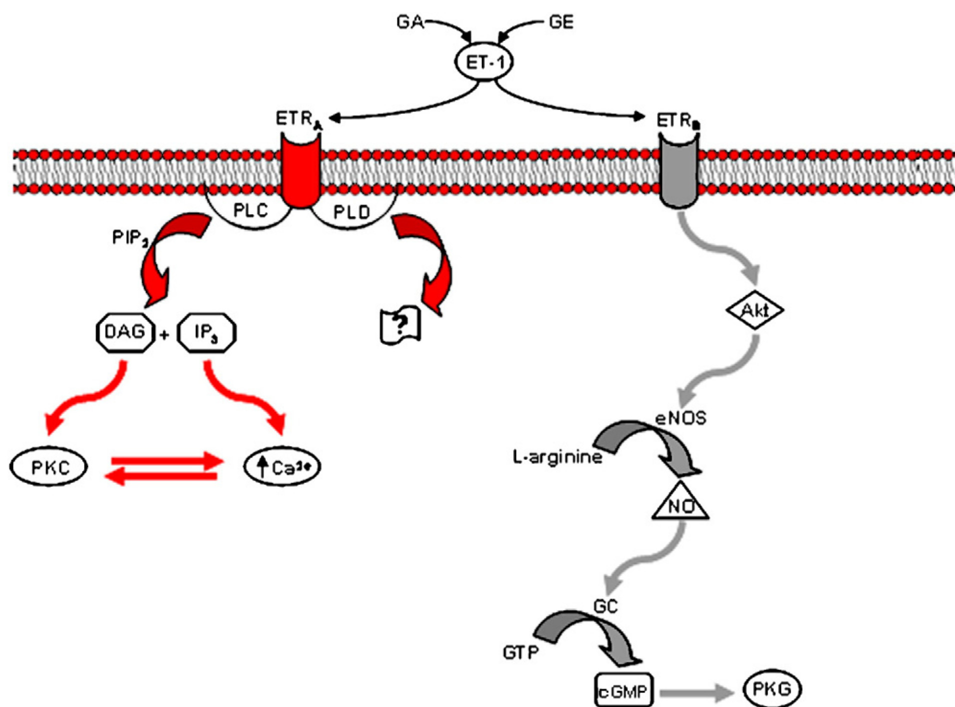

Fig. 7. Representative scheme shows the proposed intracellular signaling of GA and GE in the rat heart.

inhibitor U73122 reversed GA-induced inotropic and lusitropic effects. On the contrary, PLC inhibition enhanced the GA-dependent vasoconstriction. It is possible to hypothesize that ETR_A activation regulates both PLC and PLD activity. Indeed, PLD activity is mainly regulated by cell surface receptors including ETR_A and plays a crucial role in the biology of vascular smooth muscle cells [17].

We also found that the ET-ETR_B pathway is involved in the cardiac action of GE which is related to the activation of eNOS-NO-cGMP-PKG signal transduction pathway since they were abolished by pretreatment with eNOS (L-NAME), soluble guanylate cyclase (ODQ) and PKG (KT5823) inhibitors. This is of note since it has been reported that at the vascular level, ET-1, by interacting with endothelial ETR_B, regulates eNOS activity and NO production [18,19].

It is known that ET-1, through ETR_B, stimulates the phosphorylation of intracellular signaling molecules such as Akt [20]. According to these findings, our results showed that the increased Akt phosphorylation observed after exposure to GE was significantly reduced by ETR_B inhibition. It is also known that the Akt phosphorylation regulates eNOS-Ser1177 phosphorylation, and this is a critical requirement for eNOS activation and NO release [21,22]. In line with this knowledge, we showed a remarkable increase of eNOS phosphorylation after GE treatment which disappeared in the presence of both BQ788 and L-NAME. On the whole, our results revealed that GE-dependent cardiotropic actions occurred through the ETR_B coupled via the EE-Akt-eNOS-cGMP-PKG pathway.

In conclusion, this study provides the first evidence that both GA and GE affect cardiac performance, inducing opposite effects. Glycyrrhizin induced vasoconstriction and positive inotropic and lusitropic effects through ETR_A/PLC axis, while GE caused vasodilation and negative inotropic and lusitropic effects by stimulating the ETR_B/NOS pathway (Fig. 7). It is unknown whether GA and GE cardiac effects are beneficial or detrimental under normal or pathological conditions. Further functional and molecular studies may clarify the therapeutic potential of these two liquorice derivatives. This may be of relevance also in relation to the possible application of natural substances as triggers of signaling pathways which may play a role in myocardial and coronary protection in the presence of cardiovascular diseases.

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