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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.

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.