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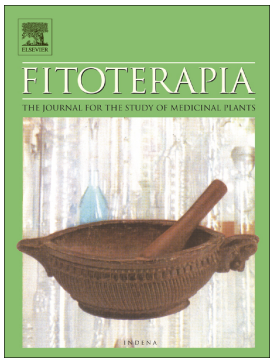

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Cardioprotective effect of 2,3-dehydrosilybin preconditioning in isolated rat heart

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Abstract

2,3-dehydrosilybin (DHS) is a minor component of silymarin, *Silybum marianum* seed extract, used in some dietary supplements. One of the most promising activities of this compound is its anticancer and cardioprotective activity that results, at least partially, from its cytoprotective, antioxidant, and chemopreventive properties. The present study investigated the cardioprotective effects of DHS in myocardial ischemia and reperfusion injury in rats.

Isolated hearts were perfused by the Langendorff technique with low dose DHS (100 nM) prior to 30 min of ischemia induced by coronary artery occlusion. After 60 min of coronary reperfusion infarct size was determined by triphenyltetrazolium staining, while lactatedehydrogenase activity was evaluated in perfusate samples collected at several timepoints during the entire perfusion procedure. Signalosomes were isolated from a heart tissue after reperfusion and involved signalling proteins were detected.

DHS reduced the extent of infarction compared with untreated control hearts at low concentration; infarct size as proportion of ischemic risk zone was 7.47 ± 3.1 % for DHS versus 75.3 ± 4.8 % for ischemia. This protective effect was comparable to infarct limitation induced by ischemic preconditioning (22.3 ± 4.5 %). Selective inhibition of Src-family kinases with PP2 (4-Amino-3-(4-chlorophenyl)-1-(t-butyl)-1H-pyrazolo[3,4-d]pyrimidine) abrogated the protection afforded by DHS.

This study provides experimental evidence that DHS can mediate Src-kinase-dependent cardioprotection against myocardial damage produced by ischemia/reperfusion injury.

Keywords

Silymarin

Dehydrosilybin

Signalosomes

Ischemia-reperfusion injury

Preconditioning

1. Introduction

Silymarin is a combination of flavonoids found in the fruit, seeds and leaves of milk thistle (*Silybum marianum* L.) including silybin, isosilybin, silydianin and silychristin [1]. The extract from seeds of this plant, and especially its major constituent silybin, is considered a hepatoprotective agent [2-4]. A pleiotropic mode of action appears to be typical for these compounds [4-6] thereby having beneficial effects in complex disorders such as metabolic syndrome [7, 8]. Besides hepatoprotective activity, silymarin has been shown to protect against ischemia-reperfusion-induced myocardial infarction [9] and oxidative stress in rat cardiac tissues [10]. Some mechanisms including anti-inflammatory, free radical scavenging, improvement of antioxidant defense systems, membrane stabilizing, iron chelating activity and inhibition of apoptosis are involved in silymarin cardiac protective effects [6]. Silymarin is, however, a mixture of polyphenolic compounds each of which may be responsible for the cardioprotective activity of the whole extract [11]. There are also minor fractions of other flavanols such as quercetin, (+)-taxifolin, and kaempferol [4, 12]. Addition of a coniferyl moiety via the hydroxyl group of its B ring yields 2,3-dehydrosilybin (DHS) [12]. We have shown that DHS displays uncoupler-like activity in isolated mitochondria [7] and attenuates reactive oxygen species formation during hypoxia/reoxygenation in primary neonatal rat cardiomyocytes [8]. We also found that DHS causes positive inotropic effect in perfused adult rat heart, the effect appears to depend on endogenous catecholamines [13]. DHS, despite demonstrating a protective effect on respiratory parameters, does not appear to uncouple H9c2 cells (permeabilised or whole), or affect mitochondrial [Ca^{++}]. On the other hand, given that it affected plasma membrane potential and whole cell [Ca^{++}], the primary site of action of DHS in whole cells was suggested to reside within the plasma membrane [14].

During ischemia, cardiomyocytes become depleted of oxygen and energy. Higher CO_2 and lactate production in the cell is associated with greater acidosis which is produced by

anaerobic metabolism. The increased acidosis increases the influx of Na^+ via the Na^+/H^+ exchanger and the depletion of ATP for NA^+/K^+ ATPase activity. The large amount of Na^+ accumulated in cytoplasm leads to the overload of Ca^{2+}. The elevated cytosolic Ca^{2+} levels contribute to the opening of a mitochondrial permeability transition pore (mPTP) and result in the death of cells [15, 16]. From metabolite point of view, succinate accumulates during ischemia leading to rapid consumption of the metabolite upon oxygen reperfusion accompanied by reactive oxygen species formation that results in cell injury [17]. Protecting the heart from these processes is the aim of many studies [18]. Preconditioning the heart to tolerate the effects of acute ischemia-reperfusion injury can be initiated through the application of several different mechanical and pharmacological strategies [19]. The protective effect of preconditioning may have been due to reduced ATP depletion and/or reduced catabolite accumulation during the sustained occlusion. The identification of the signalling pathways, which underlie the effects of this conditioning, has provided novel targets for pharmacological agents allowing one to recapitulate the benefits of these cardioprotective phenomena [20, 21]. The mechanisms of preconditioning are, in general, well studied, especially in the heart [22]. The mechanistic pathways underlying these endogenous cardioprotective phenomena are complex in nature. Cellular signalling is transduced by a complicated network of second messengers, protein kinases and other enzymes and ion channels. Signalling compartmentation is conferred in part by the formation of signalosomes [23], multimolecular protein complexes composed of unique combinations of signalling pathway components that are targeted in discrete intracellular locations via their association with anchoring or adaptor proteins [24].

In the heart, nitric oxide (NO) is known to be an important regulator of cardiac contractility in physiological condition [25, 26]. NO signalling against ischemia reperfusion injury targeted on the mitochondria is believed to be the end-target for cardioprotection. If the NO signalling

pathway is disrupted or inhibited, cardioprotection by preconditioning disappears [27]. In cardiomyocytes all 3 isozymes of NO synthase (NOS) are expressed – neuronal (nNOS, NOS1), inducible (iNOS, NOS2) and endothelial (eNOS, NOS3). NO and its signalling pathway have been shown to be important against ischemia reperfusion injury [28, 29]. Numerous studies have shown the cardioprotective effect of both endogenous and exogenous NO on ischemia reperfusion injury (summary in review [19]). eNOS generates NO which can modulate cardiovascular functions either locally or at a distance when transported as nitrite or nitroso species. Changes in shear stress, due to an increase in blood flow, are the strongest physiological stimulus of eNOS activity, which is mirrored in higher circulating NO metabolites [30].

This study presents evidence supporting the role of 2,3-dehydrosilybin as a potential therapeutic agent in case of ischemia reperfusion events, giving rise to novel applications in their prevention and treatment. It is based on data obtained using Langendorff perfusion that show better heart recovery after short exposure to 100 nM DHS. The effect is linked to formation of signalosomes that contain eNOS and is Src-kinase-dependent.

2. Materials and methods

2.1 Chemicals and test compounds

Sterile dimethylsulfoxide (DMSO), Optiprep, Percoll, 4-Amino-3-(4-chlorophenyl)-1-(t-butyl)-1H-pyrazolo[3,4-d]pyrimidine (PP2), quercetin (QUE), bradykinin (BK) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Protease inhibitor cocktail was from Roche.

The flavonolignan 2,3-dehydrosilybin was isolated from the silymarin complex (DHS, 98%; $C_{25}H_{20}O_{10}$, Mr 480) and provided by prof. Vladimír Křen at the Institute of Microbiology, Academy of Science of the Czech Republic, Prague, Czech Republic. DHS was prepared in a purity of ≥ 95% according to developed protocol based on iodine oxidation in the acetic acid with CH_3COOK [5].

The method of DHS preparation is based on base-catalyzed disproportionation [31]. For all assays, stock solutions (mM) of the compound were prepared in DMSO and further diluted with the respective solvent as required by each particular assay. DMSO content in the reaction mixtures was always kept below 0.1%.

2.2 Animals

Wistar rat males (300 g) were bred and housed in a certified animal house in accordance with European Guidelines on Laboratory Animal Care and policy of the ethics committee of the Faculty of Medicine and Dentistry, Palacký University. Animal treatment and sacrifice procedure for neonatal rats was approved by the Ethical Committee for Laboratory Animal Treatment of the Faculty of Medicine and Dentistry, Palacký University. The study was approved by the Ethical Committee for Laboratory Animal Treatment of the Faculty of Medicine and Dentistry, Palacký University, under the title "Deciphering the mechanisms of cardioprotective effects of polyphenols" and filed under number 10357/2015-3.

2.3 Perfusion protocol

The rats (male Wistar, 300 g, 10 weeks) were anesthetized with an i.p. injection of an anesthetic mixture (2% Rometar 0.5 ml + 1% Narkamon 10 ml, dose 0.5 ml solution/100 g body weight). After the i.p. heparine injection of 500 IU dose, the hearts were excised and perfused. In all experiments, the modified Langendorff method and the universal apparatus Hugo Sachs Electronic UP 100 (Germany HSE) were used. Hearts were perfused with a modified Krebs-Henseleit solution containing 118 mM NaCl, 5.9 mM KCl, 1.75 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 0.5 mM EDTA, 25 mM $NaHCO_3$, 16.7 mM glucose (pH 7.4). The perfusate was gassed with 95% O_2 - 5% CO_2, which resulted in a Po_2 > 600 mmHg at the level of the aortic cannula and a buffer pH 7.4. Treated hearts were perfused for

5 min with Krebs-Henseleit buffer (stabilization) and then with buffer containing 100 nM 2,3-dehydrosilybin (DHS) or 500 nM quercetin (QUE) or 100 nM bradykinin (BK) for 5 min or 2 μM PP2 inhibitor for 15 min, followed by 30 min ischemia and 60 min of reperfusion (see schedule; Fig. 1). Left ventricle pressure (LVP), heart rate (HR), and contractility (dP/dtmax) were measured using an analog convertor Isotec HSE, DIF modul HSE (Harvard apparatus GmbH, March-Hugstetten, Germany) [32, 33].

Fig. 1. The experimental schedule. Treated hearts were perfused for 5 min with Krebs-Henseleit buffer (stabilization) and then with buffer containing 100 nM 2,3-dehydrosilybin (DHS) or 500 nM quercetin (QUE) or 100 nM bradykinin (BK) for 10 min or 2 μM PP2 inhibitor for 15 min, followed by 30 min ischemia and 60 min of reperfusion.

2.4 Lactate dehydrogenase release assay

The activity of lactate dehydrogenase (LDH) released into the perfusate via cell membrane disruption was measured spectrophotometrically. The method is based on LDH catalysed reduction of pyruvate to lactate by an equimolar amount of NADH, the decrease of which was monitored at 340 nm [34]. For measurements, perfusate sample (50 μl) was mixed with 150 μl of fresh LDH buffer (50 mM Na_2HPO_4; 1.22 mM sodium pyruvate; pH 7.5) containing NADH (0.465 mM) on a microplate and absorbance change over time (ΔA/min) was monitored (Tecan Infinite M200, Austria). The viability of treated cells was expressed as % of control. Each experiment was performed in triplicate and independently repeated at least six times.

2.5 Measurement of infarct size (TTC staining)

After 1 h of reperfusion in the Langendorff apparatus, the hearts were perfused for 20 min in 1% triphenyltetrazolium chloride (pH 7.4, 37 °C) and after that hearts were frozen for 20 min. Hearts were cut into 2-mm-thick transverse slices from apex to base. Sections were then

immersed in Baker's formalin for 30 min at room temperature. Applying this technique, viable regions are stained red, whereas infarcted areas remain unstained. Finally, sections were photographed (Nikon D3200, objective DX AF-S Micro NIKKOR 85 mm 1:3.5G ED) and the viable and infarcted areas were measured with ImageJ software. The infarct size was expressed as a percentage of damaged area by Image J.

2.6 Mitochondrial isolation

Mitochondria from treated and untreated rat hearts were isolated immediately after Langendorff perfusion and used to isolate signalosomes. The heart was excised, washed in buffer containing 250 mM sucrose, 10 mM HEPES (pH 7.2), and 5 mM EGTA, and finely minced in the presence of 1 mg/ml protease. The minced suspension was then diluted with buffer supplemented with 0.5% fatty acid-free BSA (wt/vol) and homogenized with 4 up-and-down strokes using a rotating teflon pestle. The homogenate was centrifuged at 1700×*g* for 2 min, and the resulting supernatant was centrifuged at 9000×*g* for 5 min to collect mitochondria. The final pellet was resuspended in the initial buffer devoid of EGTA. Percoll-purified rat heart mitochondria were prepared by resuspending the pellet from the first 9000×*g* centrifugation in buffer devoid of BSA followed by centrifugation at 40000×*g* for 30 in in a self-generated 26% Percoll gradient.

2.7 Signalosome purification

To determine if caveolar bodies associated with mitochondria after tested compound treatment were present, donor mitochondria were further purified in a self-generated 24% Percoll gradient, resulting in a purified mitochondrial fraction and low-density fraction called the light layer (LL). The LL was postulated to contain signalosomes but also contained plasma membrane fragments and broken mitochondria. To purify signalosomes, we used the

nondetergent caveolae isolation protocol of Quinlan at al. [35]. Briefly, the LL fraction was adjusted to 2 ml, mixed with 50% Optiprep in buffer A (250 mM sucrose, 20 mM Tris.Cl (pH 7.8), and 1 mM EDTA), and placed at the bottom of a centrifuge tube. A 20-10% Optiprep gradient was layered on top, and tubes were centrifuged at 52 000×*g* for 90 min. After centrifugation, the top 5 ml were collected and mixed with 4 ml of 50% Optiprep solution. This mixture was then overlayed with 5% Optiprep solution and centrifuged again at 52 000×*g* for 90 min. The signalosome fraction was identified as an opaque band at the 5% interface.

2.8 Western immunoblotting

Total proteins were prepared from signalosome fraction in RIPA buffer (in mM: 150 NaCl, 10 Tris, 5 EDTA, 0,1 Na_3VO_4, 1 NaF, 1% sodium deoxycholate, 10% SDS, 1% Triton X-100, 1 protease inhibitor cocktail tablet) and incubated at 4 °C for 30 min. The extracts were later centrifuged at 12 000×*g* at 4 °C for 15 min. Equal amounts of total protein (20 µg) were boiled and separated with SDS-PAGE and electrophoretically transferred to a PVDF membrane. Membranes were blocked with Tris-buffered saline-Tween 20 buffer (TBST) containing 5% non-fat milk at room temperature for 2 h, and then incubated overnight at 4 °C with primary antibodies. The primary antibody dilutions were 1:1000 for eNOS (rabbit, DAM 1424997, MerckMillipore). The membrane was then washed three times with TBST buffer and incubated in TBST buffer containing 5% non-fat milk with secondary antibody (diluted 1:10000, Santa Cruz) for 1 h at room temperature. Finally, the membrane was washed with TBST three times. For detection, the membranes were saturated with an enhanced chemiluminescence mixture for 1 min and viewed by autography using preflashed X-ray film (Kodak Scientific Imaging film) for 60 s. The bands were analysed by densitometric scanning using ImageJ software.

2.9. Statistical analysis

Where applicable analysis of variance (ANOVA) with post-hoc Tukey test was applied using STATISTICA software (StatSoft, Tulsa, OK, USA). $p < 0.05$ was considered statistically significant.

3. Results

The isolated Langendorff-perfused heart method has many advantages. It enables several sets of experiments, standardized conditions during reperfusion and permits interventions both prior to removal of the heart and during the perfusion before or after ischemia [36]. We used the preconditioning model, in which the tested compound was added to the buffer prior to ischemia for 5 min. After 30 min of ischemia, when the heart perfusion was stopped and the heart was dipped into a prewarmed Krebs-Henseleit buffer (37 °C), 60 min of reperfusion followed. Physiological parameters such as left ventricle pressure, heart rate, contractility and blood flow were monitored during the experiment (vide infra).

We selected the 100 nM DHS concentration as optimum based on our previous results [13] that showed concentration of 1 μM DHS and higher caused inotropic effect that ultimately led to heart damage. Our pilot experiments showed that 10 nM DHS had no beneficial effect.

3.1. DHS protects the heart against ischemia/reperfusion injury in perfused rat hearts

Basing the tested concentration on our previous work in perfused hearts [13], we used here preconditioning perfusion model to observe the effect of 100 nM DHS on the whole organ. DHS was added for 5 min before 30 min of ischemia. Following reperfusion, i.e. restoration of perfusate flow, all biomechanical parameters rapidly returned to values observed in control non-ischemic hearts (Fig. 2; ▲, dotted line). We decided to begin an investigation of the

signaling pathways involved in DHS protection by comparing it with the well-characterized effects of bradykinin. Physiological effect of bradykinin is vasodilation accompanied by lower blood pressure, in ischemic tissue it acts as an endogenous cytoprotective mediator. Bradykinin exerts cardioprotective effect in ischemic preconditioning via activation of PI3K/Akt/eNOS signaling pathway and regulation of redox state via NO release [37]. We used 100 nM bradykinin (Fig. 2; ●, dashed line) and confirmed that bradykinin preconditioning elicits cardioprotective effects against postischemic contractile dysfunction similar to that of ischemic preconditioning. Studies focused on the efficiency of flavonoids against ischemia-reperfusion (I/R) injury have demonstrated that quercetin exerts robust protective effects in renal, cerebral, and hepatic ischemia reperfusion models [38]. 500 nM quercetin (Fig. 2; ∗, dashed line) was tested to compare its effect with DHS. Quercetin treatment exerts significant positive effects on isolated hearts but its effect is weaker than that of DHS within the time period evaluated (Fig. 2). These results are consistent with the beneficial effects of DHS and other flavonoids on the cardiovascular system.

Fig. 2. Effect of DHS on biomechanical parameters in perfused adult rat heart. Isolated adult rat hearts were perfused with Krebs-Henseleit buffer for 5 min followed by 10 min perfusion with a chosen compound or Krebs-Henseleit buffer in control hearts, 30 min of ischemia and concluded by 60 min of reperfusion (wash-out) with Krebs-Henseleit buffer. Data presented were obtained for control hearts (■, full line), ischemia hearts (◆, dashed line) and hearts treated with: 100 nM DHS (▲, dotted line); 500 nM QUE (∗, dashed line) and 100 nM BK (●, dashed line). Individual panels are: A – left ventricle pressure; B – heart rate; C – contractility; D – blood flow. Data are average ± SD for four independent experiments, <0.05 * vs ischemia group.

Several members of the Src tyrosine kinases family were found to reside in mitochondria. Src tyrosine kinase is tightly linked to reduced cell injury in preconditioned cardiac mitochondria [39]. Cardioprotection by bradykinin involves Src kinase, endothelial nitric oxide synthase, guanylyl cyclase, protein kinase G (PKG), mitochondrial ATP-sensitive potassium channel (mitoK_{ATP}), and ROS. Participation of these elements was evidenced by the data published by Pasdois et al. [40], where bradykinin protection against cardiac dysfunction and infarction is shown to be abolished by PP2, a Src kinase inhibitor. To investigate the mechanisms of PP2-induced decrease and attenuation of DHS stimulation, we studied effects of PP2 on the biomechanical parameters in isolated hearts by Langendorff perfusion (Fig. 3). We applied a widely used selective inhibitor of Src tyrosine kinases, PP2 (2 μM; 4-Amino-3-(4-chlorophenyl)-1-(t-butyl)-1H-pyrazolo[3,4-d]pyrimidine), before ischemia for 15 min (Fig. 3; dashed line). Interestingly, PP2 preconditioning alone improved all biomechanical parameters during reperfusion. We also tested PP2 in the presence of 100 nM BK to confirm that PP2 blocks BK targeting of Src-dependent signalling pathway (Fig. 3; ●, dotted line). Indeed, the BK effect was significantly diminished, except in the case of blood flow. Similarly, PP2 diminished the effect of DHS significantly. However, the inhibition of DHS effect by PP2 was not profound as in the case of BK (Fig. 3). For better clarity and comparison the effect of DHS in the presence and absence of PP2 is shown in Supplemental Figure 1.

Fig. 3. Effect of DHS+PP2 on biomechanical parameters in perfused adult rat heart. Isolated adult rat hearts were perfused with Krebs-Henseleit buffer for 5 min followed by 15 min perfusion with PP2 or combination of PP2 with tested compound or Krebs-Henseleit buffer in control hearts, 30 min of ischemia and concluded by 60 min of reperfusion (wash-out) with Krebs-Henseleit buffer. Data presented were obtained for control hearts (■, full line), PP2 inhibitor alone (◆, dashed line) and hearts treated with PP2 in the presence of: 100

nM DHS (▲, dotted line), 100 nM BK (●, dashed line) and 500 nM QUE (∗, dashed line). Individual panels are: A – LVP; B – heart rate; C – contractility; D – blood flow. Data are average ± SD for four independent experiments, <0.05 * vs PP2 group.

3.2 DHS modulates cardiomyocyte viability and LDH release

We monitored cell viability by LDH activity in the perfusate to evaluate the general effects of ischemia and reoxygenation on perfused heart. Although CPKMB is a more specific marker in case of blood testing, we have used a detection of LDH activity in perfusates. It was confirmed that LDH activity is an able marker for diagnosis of ischemic heart disease [1, 6, 12]. In our case we measured LDH activity only in samples from isolated heart tissue where the activity is comparable with CKMB [1].

A significant increase of LDH released was detected in the ischemia group (ischemia vs control group; *p < 0.05; Fig. 4A). LDH activity in the perfusate was reduced during the reperfusion phase if hearts were preconditioned with 100 nM DHS, 100 nM bradykinin (BK) and also 500 nM quercetin (QUE). Each treatment significantly diminished the ischemia-induced LDH release during the first ten minutes of reperfusion (Fig. 4A). The potency of each treatment differed with BK being the most powerful and QUE the weakest.

Figure 4B shows that LDH activity detected in the perfusate was much lower if PP2 was used in the experiment.

Fig. 4. LDH release from cells during perfusion. A. DHS decreases LDH release in reperfusion. **B.** Tested compounds with combination with PP2 inhibitor. Data presented were obtained for control hearts (■, full line), ischemia hearts (◆, dashed line) and hearts treated with: 100 nM DHS (▲, dotted line); 500 nM QUE (∗, dashed line) and 100 nM BK (●, dashed line). Data are average ± SD for four independent experiments, A <0.05* vs ischemia group, B <0.05* vs PP2 group.

3.3 DHS affects infarct size in the hearts after ischemia/reperfusion

TTC staining is the method of choice in our laboratory for postmortem determination of infarct size. The viable myocardium is stained as the water-soluble compound TTC is converted by active mitochondrial dehydrogenases into an insoluble red precipitate [41]. The heart is immersed in neutral TTC solution for 15 min to 20 min at 37°C and then sliced at a thickness of 1 mm. The extent of staining correlates with the number of viable mitochondria and differentiates viable and nonviable tissue. TTC staining demonstrates that 30 min of global ischemia followed by 1 h of reperfusion in the untreated heart brought about the infarction of 76.6 % of the area at risk ("Ischemia" in Fig. 5). DHS and BK treatment reduced the infarct size was reduced by 60 % and 50%, respectively, with respect to that of untreated hearts (*$p < 0.05$). QUE had much lower although still significant effect on the infarct size. Of note is the non-negligible infarct area in the control hearts that did not undergo ischemia as compared to sham hearts that were perfused with TTC immediately upon removal, i.e. were not perfused using Langendorff apparatus.

Fig. 5. Viability of heart – TTC staining combined with ImageJ. (A) TTC staining was used to assess myocardium infarct area after different interventions. Representative sections of myocardium - red areas represent non-ischemic rat heart issue; white areas represent ischemic rat heart issue. (B) Quantitative analysis of infarct area (%). More details in Methods 2.5. Data are average ± SD for four independent experiments, <0.05* vs control, <# vs ischemia.

The Src kinase inhibitor PP2 treatment does not modify the infarct size by itself, which is interesting when effect on LDH release is considered (vide supra), but blocks protection

against contractile failure and infarction after ischemia-reperfusion injury. In these respects, the effects of DHS were the same as the effects of BK and QUE (Fig. 6).

Fig. 6. Viability of heart – TTC staining combined with ImageJ. (A) TTC staining was used to assess myocardium infarct area after different interventions (PP2 inhibitor). Representative sections of myocardium - red areas represent non-ischemic rat heart issue; white areas represent ischemic rat heart issue. (B) Quantitative analysis of infarct area (%). More details in Methods 2.5. Data are average ± SD for four independent experiments, <0.05* vs PP2 group.

3.4. Involvement of signalosomes in cardioprotection induced by DHS

Cellular processes such as cardiac myocytes contractility and hypertrophy are regulated by a complex intracellular network of signal transduction pathways [42]. It has become appreciated in recent years that several mechanisms exist in cells to retain signalling specificity while permitting pleiotropism, including the physical separation and spatial compartmentation of different pathways [24, 43]. Signalling compartmentation is conferred of unique combinations of signalosomes, multimolecular protein complexes composed of unique combinations of signaling pathway components that are targeted in discrete intracellular locations via their association with anchoring or adaptor proteins [23]. The co-localisation of pathway components permits the efficient transmission of signals between adjacent molecules while avoiding inappropriate cross-talk with other pathways in the cells [24].

Mitochondria and purified LLs from donor hearts perfused with medium containing Krebs-Henseleit buffer or tested compounds were obtained as described in paragraph 2.7. As shown in Fig. 7, eNOS, a known component of bradykinin signal transduction [44], was found to be enriched in the LL fraction isolated from DHS treated hearts. Remarkably, in the presence of

PP2 higher eNOS content of signalosomes was detected in the DHS treated hearts as compared to untreated ischemic hearts. Of note is also our finding that LL fraction from BK treated hearts contained the same amount of eNOS regardless of PP2 presence.

Fig. 7. Detection of eNOS after perfusion of heart and signalosomes isolation. Immunodetection analysis of DHS-treated signalosomes. A representative immunoblots of four independent experiments. 10 μg of LLs from untreated (sham), control or DHS-treated hearts, obtained as described in Materials and Methods, were loaded. eNOS, endothelial nitric oxide synthase. B. ImageJ software was used for pixel density analysis. Data are average ± SD for four independent experiments, <0.05* vs sham, <0.05# vs compounds untreated with PP2.

4. Discussion

An overall aim is to understand what causes the damage to the heart when ischemia is present in order to understand how to prevent or to cure. Many papers showed positive effect of natural compounds on human health but only few explain the mechanism of this effect or a pathway, which is involved during the protective processes [20, 45]. Our study evaluates the cardioprotective effect of silymarin component, 2,3-dehydrosilybin (DHS), in isolated heart model. We show that preconditioning with 100 nM DHS is able to improve biomechanical parameters in comparison with the untreated heart, limit infarct size associated with ischemia reperfusion injury and limit LDH release. The last two effects are obviously linked because the bigger the injury (infarct size), the more LDH is released. Concentration of DHS used in our experiments, 100 nM, does not support the possibility of LDH inhibition or maintenance of cytosolic membrane integrity. Rather, it suggests DHS acting on the level of a signaling

pathway.

Our previous work demonstrated inotropic effect of DHS in perfused heart at concentrations from 100 nM and higher, with 10 μM being the highest and most deleterious. Follow up study concluded that DHS has the primary site of action at the plasma membrane [14]. Taken together, the data suggest a low concentration of DHS could be effective, triggering only mild stimulation of heart activity, while acting on plasma membrane level. It leads to a comparison with either hormone or hormone-like effectors, e.g. bradykinin, or artificial uncouplers. But uncouplers fit not as we already postulated that DHS is unlikely to act as an uncoupler [14]. Therefore bradykinin was our obvious choice.

Bradykinin used at equal concentration as DHS displayed positive effect on heart biomechanical parameters (Fig. 2). Some parameters were affected more by DHS, some by bradykinin, but overall impression leads one to the hypothesis that both may act through the same or similar signaling pathway. However, bradykinin is a potent vasodilator that results in blood pressure lowering and DHS, according to our data, increases left ventricle pressure and heart contractility thereby mimicking (nor)epinephrine. Considering the effect of bradykinin on signalosomes formation and the content of signalosomes [40], we endeavored into modulation of bradykinin and DHS effect by the Src-kinase family inhibitor, PP2 (2 μM).

In both cases the cardioprotective effect was diminished, profoundly in the case of bradykinin, much less in the case of DHS (Fig. 3). This suggests high dependency of bradykinin effect on Src-kinase and only partial dependency of DHS on the same. Hence we aimed to distinguish the culprit.

Myocardial ischemia reperfusion is accompanied by the release of structural proteins, enzymes and other intracellular molecules into the cardiac interstitium [46]. Therefore we assessed cell viability was using TTC staining of heart tissue and detection of enzymes in perfusates, namely LDH. Cytosolic enzymes, including LDH, may be used as diagnostic

markers of myocardial ischemia injury, as they are released from the damaged myocardial tissues into the blood serum when cell membrane was induced to permeate or rupture [47, 48]. Therefore, LDH levels in perfusates reflected changes in membrane integrity and the extent of myocardial injury. This study confirmed that pretreatment with both BK and DHS reduced the elevated levels of LDH induced by ischemia reperfusion injury, suggesting that both trigger events that result in reduced cell membrane damage in myocardial ischemic injury. In the same venue, TTC staining, which shows metabolic capacity rather than just integrity of a cell, displayed cardioprotective effect of both DHS and BK. In all cases the presence of PP2 diminished the positive effect. Surprisingly, PP2 alone had positive effect on the ischemia reperfusion damaged tissue suggesting that Src-kinase-dependent signaling plays a dual role. However, this was not the aim of our study and we leave this interesting question to others to resolve.

Endothelial dysfunction is a main event in the pathogenetic cascade conducing to cardiovascular events [49] and therapies aiming at preserving endothelium are needed for the effective prevention of cardiovascular disease [50]. NO is a free radical produced by the NOS-catalyzed oxidation of arginine to citrulline. NO is well-established as a trigger and mediator for cardioprotection [51, 52]. Yan-yan et al. [53] investigated the protective effects of *Shenfu* injection (SFI) against myocardial ischemia/reperfusion injury in model rats and explored its mechanism of action. The phosphorylation of eNOS and the concurrent increase of NO production contributed significantly to the protective effects of *Shenfu*.

Because eNOS is one of the proteins participating in signalosome-dependent signalling we tested its presence in light layer (LL) isolated from treated and untreated hearts. Our data show stronger eNOS presence in LL from DHS treated hearts than in BK treated hearts. While PP2 affects the level of eNOS in LL from DHS treated hearts, it does not affect the same in BK treated hearts suggesting this is the difference between both effectors. Therefore, it

appears DHS improves endothelial activity and the effect is partially dependent on Src-kinase. ADMA, an endogenous inhibitor of nitric oxide synthase (NOS), plays a pivotal role in endothelial dysfunction. In the study of Li Volti et al. [50] silibinin markedly improves endothelial dysfunction in db/db obese mice by reducing circulating and vascular asymmetric dimethylarginine (ADMA) levels. Therefore silibinin has a positive effect on endothelial function and implicitly on cardiovascular homeostasis. Because silibinin is the parent drug of DHS, we could speculate that DHS affects the endothelial functions in the heart such that it can cope with ischemia reperfusion events.

5. Conclusion

In this study, 2,3-dehydrosylibin (DHS) as a remedy for cardiac diseases was evaluated. We hypothesized that DHS would limit infarct size and assessed the possible cardioprotective effects in a rat isolated heart model of coronary artery occlusion and reperfusion with infarct size as the endpoint of ischemia-reperfusion injury. Our data support the role of DHS in modulation of endothelial function in the heart.

Conflict of interest

None

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