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ORIGINAL ARTICLE

Isoespintanol, a monoterpene isolated from oxandra cf xylopioides, ameliorates the myocardial ischemia-reperfusion injury by AKT/PKCε/eNOS-dependent pathways

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Abstract

Purpose To determine the actions of isoespintanol (Isoesp) on post-ischemic myocardial and mitochondrial alterations.

Methods Hearts removed from Wistar rats were perfused by 20 min. After this period, the coronary flow was interrupted by half an hour and re-established during 1 h. In the treated group, Isoesp was administered at the beginning of reperfusion. To assess the participation of ε isoform of protein kinase C (PKCε), protein kinase B (PKB/Akt), and nitric oxide synthase (NOS), hearts were treated with Isoesp plus the respective inhibitors (chelerythrine, wortmannin, and N-nitro-L-arginine methyl ester). Cell death was determined by triphenyl tetrazolium chloride staining technique. Post-ischemic recovery of contractility, oxidative stress, and content of phosphorylated forms of PKCε, Akt, and eNOS were also examined. Mitochondrial state was assessed through the measurement of calcium-mediated response, calcium retention capacity, and mitochondrial potential.

Results Isoesp limited cell death, decreased post-ischemic dysfunction and oxidative stress, improved mitochondrial state, and increased the expression of PKCε, Akt, and eNOS phosphorylated. All these beneficial effects achieved by Isoesp were annulled by the inhibitors.

Conclusion These findings suggest that activation of Akt/eNOS and PKCε signaling pathways are involved in the development of Isoesp-induced cardiac and mitochondria tolerance to ischemia-reperfusion.

Keywords Isoespintanol · Ischemia-reperfusion · Infarct size · Akt · PKCε · eNOS

Abbreviations

Isoesp	Isoespintanol
NOS	Nitric oxide synthase
L-NAME	L-N^G-Nitroarginine methyl ester
PKCε	Protein kinase C ε
Che	Chelerythrine
Akt	Protein kinase B
Wort	Wortmannin
DMSO	Dimethyl sulfoxide
CR	Coronary resistance
CPP	Coronary perfusion pressure
CF	Coronary flow
GSH	Reduced glutathione
TBARS	Thiobarbituric acid reactive substances
LSD	Light scattering decrease
CRC	Ca^{2+} retention capacity
ΔΨ	Mitochondrial membrane potential
mPTP	Mitochondrial permeability transition pore
TTC	Triphenyltetrazolium chloride
LVDP	Left ventricular developed pressure
$+dP/dt_{max}$	Maximal velocity of rise of left ventricular pressure
LVEDP	Left ventricular end diastolic pressure
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
ROS	Radical oxygen species

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Introduction

Cardiovascular diseases are chronic diseases responsible for the highest morbidity and mortality worldwide being the coronary artery disease or ischemic heart disease (IHD) the most common type. IHD refers to a clinical state characterized by low coronary blood flow arising from various cause (hypertension, smoking, diabetes, physical inactivity, obesity, etc.) but resulting in a lack of myocardial oxygen supply (Murray and Lopez, 2013). This event produces myocyte death or infarction and cardiac contractility dysfunction. Although reperfusion is mandatory to salvage ischemic myocardium, it exacerbates the damage increasing the infarct size. Over the last years, several studies have shown that excessive generation of reactive oxygen species (ROS) and calcium overload lead to activation of deleterious signaling pathways, which aggravate the myocardial ischemia-reperfusion injury (Murphy & Steenbergen, 2008). On the other hand, increasing lines of evidence suggest that myocardial injury is accompanied by mitochondrial dysfunction principally associated to formation of permeability transition pore (mPTP) (Morciano et al. 2017). Thus, mitochondrial preservation through multiple kinase-dependent pathways appears as the principal mechanism responsible of the improvement of myocyte viability achieved by "cardioprotective" interventions (Boengler et al. 2018; Kleinbongard and Heusch 2015; Davidson et al. 2019).

Unfortunately, current therapies have provided poor clinical outcomes and the identification of new pharmacological agents emerges as a promising approach for IHD treatment. Herbal products were used as conventional medicines for thousands of years and numerous investigations recognized its beneficial effects against a large variety of cardiac disorders (Li et al. 2015; Newman and Cragg, 2016; Shukla et al. 2010, Liperoti et al. 2017; González Arbeláez et al. 2018). At experimental level, previous data from our laboratory show reduction of infarct size and improvement of post-ischemic recovery of myocardial contractility after treatment with plant-derived aqueous extracts (Lopera et al. 2013; González Arbeláez et al. 2016a,b; Fantinelli et al. 2017). These studies also present evidence about the participation of PI3K/Akt and NO-dependent pathways in the beneficial effects afforded by the extracts.

Terpenes are natural phytochemicals present in human diet and widely used in food industries, and cosmetic and pharmaceutical sectors. Among the molecules with promising pharmacological activities, monoterpenes have shown a remarkable cardioprotective effect against ischemia-reperfusion injury (Santos et al. 2011; Britto et al. 2018; Chen et al. 2015; Chen et al. 2017; Nagoor Meeran et al. 2016).

Isoespintanol is a monoterpene firstly extracted from *Eupatorium saltense* and later isolated and identified in leaves of *Oxandra xylopioides* (Rojano et al. 2007). Previous studies demonstrated the anti-inflammatory (Rojano et al. 2007), anti-parasitic (Hocquemiller et al. 1991), antioxidant (Rojano et al. 2008), and anti-spasmodic actions of isoespintanol (Gavilánez Buñay et al. 2018). In spite of these multiple biological actions, the effects of isoespintanol in the heart during ischemia-reperfusion have not yet been described.

Thus, the present study was designed to explore cardiovascular effects of isoespintanol examining its actions on infarct size, contractile function, mitochondrial alterations, and mechanisms involved when perfusate flow to entire heart was stopped by 30 min (global ischemia) and restarted by 60 min (reperfusion).

Material and methods

Isoespintanol

Isoespintanol (Isoesp) isolation and dissolution were previously described by Rojano et al. (2007). The purity of this compound was determined by a gas chromatography (6890 N)–mass spectrometry (5973 N), equipped with a split/splitless injector (Agilent Technologies, Santa Clara, California, USA). Isoesp retention time was 21.4 min and purity > 99% (Fig. 1a). Database NIST 98 was used to identify the compounds. The GC-MS spectrum (Fig. 1b) shows the different fragments of the sample.

Animals

Male Wistar rats were used. Animals were housed 4 per cage with food and drinking water "ad libitum." The room ventilation rate was 4–6 changes per hour, at temperature of 22 ± 2 °C and with a light cycle/dark of 12 h. All procedures followed during this investigation were approved by the Animal Welfare Committee of La Plata School of Medicine (University of La Plata), following the Guide for the Care and Use of Laboratory Animals published by the National Research Council, National Academy Press, Washington DC 2010, and/or European Union Directive for Animal Experiments 2010/63/EU (P01-05-2015). The animals were assigned to the following experimental designs:

Isolated perfused heart

Rats were anesthetized with an intraperitoneal injection of urethane (0.6 ml/100 g). Central thoracotomy and heart excision were performed immediately after phase III anesthesia was reached, verified by the loss of pedal withdrawal reflex. The rat's chest was opened and the heart was rapidly isolated, transferred to Langendorff apparatus, and perfused through

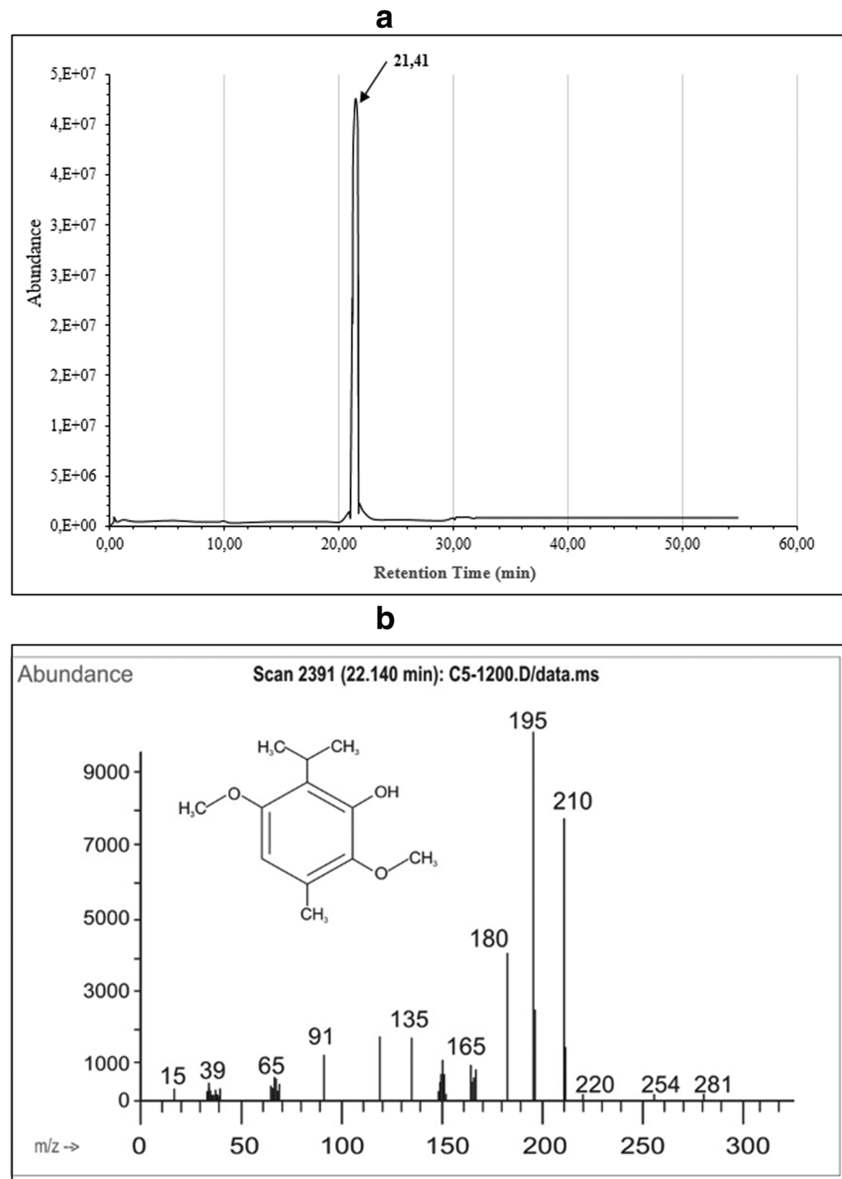

Fig. 1 The chromatogram (**a**) and mass spectrometry (**b**) show the isolation of isoespintanol and the mass analysis of sample components

aorta under a perfusion pressure (CPP) of 60 to 70 mmHg. Coronary flow (CF) was 11 ± 2 mL/min and was maintained during all the experiments. The perfusion solution containing (in mmol/L) 118 NaCl, 5.9 KCl, 1.2 $MgSO_4$, 1.35 $CaCl_2$, 20 $NaCO_3H$, and 11.0 glucose was prepared daily and continuously oxygenated with 95% O_2 and 5% CO_2 and maintained at pH 7.4 and temperature of 37 °C. Heart was paced at 280 ± 10 beats/min. A latex balloon was connected to a Statham P23XL pressure transducer and inserted to the left ventricle through the mitral valve. The balloon was filled with water to achieve a left ventricular end-diastolic pressure (LVEDP) between 8 and 12 mmHg and this volume was unchanged during the experiment. Left ventricular pressure (LVP) and CPP data were acquired by using an analog-to-digital converter and acquisition software (Chart V4.2.3 AD Instruments).

Experimental protocols

The protocols appear in Fig. 2. In the first group (NIC: non-ischemic control), hearts were perfused during 90 min; in the second group (IC: ischemic control), the coronary flow was interrupted by half an hour and re-established by 1 h. Other hearts received Isoesp 0.6 μg/mL or dimethyl sulfoxide (DMSO) 0.6 μg/mL during the first 10 min of reperfusion. In additional groups, L-NAME (L^G-nitro-L-arginine methyl ester, NOS inhibitor) 1 mM or chelerythrine (PKC inhibitor) 1 μM or wortmannin (PI3K/Akt inhibitor) 1 mM was administered simultaneously and previous to Isoesp treatment.

Separated groups of hearts subjected to the same protocols were used to measure lipid peroxidation and reduced glutathione content and to perform western blotting analysis.

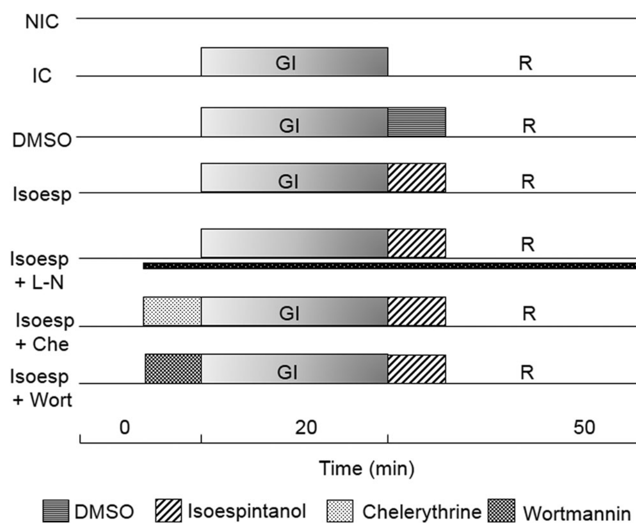

Fig. 2 Scheme of the experimental protocols. NIC: non-ischemic control; IC: ischemic control; Isoesp: Isoespintanol; Isoesp + L-N: L^G-nitroL-arginine methyl ester, inhibitor of NOS; Isoesp + Che: chelerythrine, inhibitor of PKC and Isoesp+Wort: wortmannin, inhibitor of PI3K/Akt

Measured parameters

Infarct size Infarct size was assessed by triphenyltetrazolium chloride (TTC, Sigma-Aldrich, Munich, Germany) staining technique. At the end of reperfusion, atrial and right ventricular tissues were excised and left ventricle (LV) was frozen. After, LV was cut into six transverse slices, which were incubated for 5 min at 37 °C in a 1% solution of TTC. To measure myocardial infarction, the slices were weighed and scanned. Infarcted (pale) and viable ischemic/reperfused (red) areas were measured by computed planimetry (Scion Image 1.62; Scion Corp., Frederick, Maryland, USA). Infarct weights were calculated as (A1 × W1) + (A2 × W2) + (A3 × W3) + (A4 × W4) + (A5 × W5) + (A6 × W6), where A is the area of infarct and W is the weight of the respective section. Infarct size was expressed as a percentage of total area (Suzuki et al. 2002).

Cardiac function Systolic function was assessed through left ventricular developed pressure (LVDP) and maximum velocity of LV pressure rise ($+dP/dt_{max}$). Diastolic function was evaluated by left ventricular end diastolic pressure (LVEDP) and maximum velocity LV pressure decay ($-dP/dt_{max}$).

Coronary resistance Coronary resistance (CR) was calculated as CPP/CF ratio.

Oxidative stress a. Lipid peroxidation

Thiobarbituric acid reactive substance (TBARS) used as a marker for lipid peroxidation was detected by spectrophotometric method (González Arbeláez et al. 2016) and expressed in nmol × mg^{-1} protein using an extinction coefficient of 1.56 × 10^5 M^{-1} cm^{-1}.

b. Reduced glutathione (GSH)

GSH was determined by Ellman's method (González Arbeláez et al. 2016) and expressed in μg × mg^{-1} protein.

Western blotting Proteins were extracted from LV in RIPA lysis buffer. The samples were separated on a 10% SDS-polyacrylamide gel, transferred to PVDF membranes, and blocked. After, membranes were incubated overnight at 4 °C with primary antibodies: phospho-Akt Ser473, phospho-PKCε, phospho-eNOS Ser1177, and 3-nitrotyrosine. Blots were washed and incubated with horseradish peroxidase (HRP)–conjugated secondary antibody. Densities of protein bands were detected by chemiluminescence.

Isolated mitochondria

Fresh cardiac mitochondria were obtained by differential centrifugation (Mela and Seitz, 1979). Briefly, LV was washed and homogenized (in presence of proteinase) in ice-cold solution. After, homogenate was centrifuged at 750×*g* and supernatant was centrifuged at 8000×*g* to sediment mitochondria. This pellet was washed and finally re-suspended in a cold solution containing mannitol and sucrose. Purity of preparation was determined by immunodetection of mitochondrial outer membrane voltage-dependent anion channel (VDAC) and by absence of GAPDH (Soetkamp et al. 2014).

Measured parameters

Calcium-mediated response To assess the response to Ca^{2+}, 0.3 mg of mitochondrial protein/milliliter was incubated during 5 min in a respiration buffer containing (in mmol/L) the following: 120 KCl, 20 MOPS, 10 Tris HCl, and 5 KH_2PO4 adjusted to pH = 7.4. After 5-min preincubation, in the mitochondria energized with the addition of 5 mmol/L succinate, it was assessed the resistance to mPTP opening with 100 μmol/L $CaCl_2$. If mPTP is open, solutes will be free to enter the inner matrix causing mitochondria to swell. The change is observed as light scattering decrease (LSD) and followed at 520 nm (Baines et al. 2003). LSD was assessed in fresh mitochondrial samples without any treatment and in the presence of Isoesp 0.6 μg/mL. In order to relate LSD to mPTP opening, we added cyclosporine A 0.5 μM (mPTP inhibitor) to samples of mitochondria.

Calcium retention capacity This assay measures the amount of Ca^{2+} that mitochondria can accumulate and retain before the precipitous release that marks mPTP opening. Extramitochondrial Ca^{2+} fluxes are measured fluorometrically at 506 and 532 nm as excitation and emission wavelengths using Calcium Green 5N. Mitochondria (0.3 mg/mL) are suspended in 2 mL of

respiration buffer with 0.5 μM Calcium Green 5N. The suspension is subjected to a train of Ca^{2+} pulses of 10 μM. Each pulse represents the increase in fluorescence (Ca^{2+} binding to Calcium Green 5 N) followed by a decrease in fluorescence due to Ca^{2+} influx into mitochondria. After sufficient Ca^{2+} loading, extramitochondrial Ca^{2+} concentration abruptly increased, indicating a massive release of Ca^{2+} by mitochondria due to mPTP opening. Calcium retention capacity (CRC) was measured in fresh mitochondrial samples without and with Isoesp 0.6 μg/mL and expressed as nmol Ca^{2+}/mg of protein (Pardo et al. 2015).

Mitochondrial potential Mitochondrial potential (ΔΨm) changes were evaluated by measuring rhodamine-123 (RH-123) fluorescence quenching under the buffer containing RH-123 0.1 μM and at 503 and 527 nm as excitation and emission wavelengths. During the measurements, the reaction medium containing mitochondria (0.1 mg/mL) was continuously stirred. RH-123 uptake is in proportion to ΔΨm and the rate of fluorescence quenching is a function of ΔΨm. Finally, ΔΨm was calculated using Nernst-Guggenheim equation (Pardo et al. 2015).

Statistical analysis

Data were expressed as mean ± SEM and evaluated with either unpaired Student's *t* test or one- or two-way ANOVA followed by Bonferroni post hoc test, to compare differences among groups. A p value < 0.05 was considered significant.

Results

TTC measurement

The interruption of coronary flow by half an hour and its re-establishment by 1 h produced an infarct size of approximately 30%. This value was not modified by DMSO. Isoesp treatment decreased infarct size reaching a value of approximately 10%. This Isoesp-mediated beneficial effect was abolished when eNOS, PKCε, or PI3K/Akt were inhibited (Fig. 3). The addition of inhibitors to ischemic control hearts did not modify the infarct size observed in absence of them (data not shown).

Hemodynamic parameters

Hemodynamic parameters are shown in Fig. 4 a, b, and c. In IC- and DMSO-treated hearts, post-ischemic recovery of myocardial function was scarce and LVDP, $+dP/dt_{max}$, and $-dP/dt_{max}$ reached values of approximately 20% of baseline at the end of reperfusion. Hearts treated with Isoesp exhibited a significant increase of those parameters detecting values of approximately 70% of pre-ischemic value at 60-min reperfusion. LVEDP increased during reperfusion in IC- and DMSO-treated hearts and this increase was significantly attenuated by Isoesp treatment. The inhibition of NOS, PKC, and PI3K/Akt annulled Isoesp actions and hemodynamic parameters reached similar values to those obtained in IC- and DMSO-treated hearts.

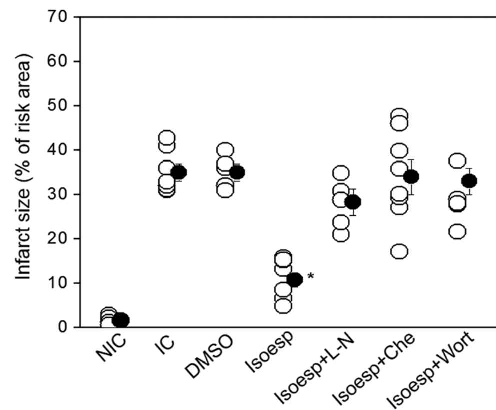

Fig. 3 Infarct size (IS), expressed as a percentage of risk area, in ischemic control (IC, $n = 8$) and in hearts treated with dimethyl sulfoxide (DMSO, $n = 5$) or isoespintanol (Isoesp, $n = 8$) or Isoesp + L-N ($n = 5$), Isoesp + Che ($n = 8$), and Isoesp + Wort ($n = 6$). Observe that Isoesp treatment decreased the IS obtained in IC hearts (10% vs. 30%, approximately). This beneficial action of Isoesp was abolished by NOS inhibition (IS ~ 28%), PKC inhibition (IS ~ 34%), or PI3K/Akt inhibition (IS ~ 33 %). Statistical test: one-way ANOVA with a Bonferroni multiple comparison post hoc test. $^{*}p < 0.05$ vs. IC or DMSO; $^{\#}p < 0.05$ vs. Isoesp

Coronary resistance

At the end of reperfusion, the increase of CR detected in IC- and DMSO-treated hearts was not observed after Isoesp treatment. This beneficial action was abolished when Isoesp was infused in presence of L-N, Che, or Wort (Fig. 4d).

GSH, TBARS, and 3-nitrotyrosine

GSH content decreased after ischemia-reperfusion in IC and DMSO groups but it was significantly preserved by Isoesp. When Isoesp was administered in the presence of NOS, PKC, and PI3K/Akt inhibition, GSH acquired similar values to those observed in IC- or DMSO-treated hearts (Fig. 5a). TBARS concentration—as an index of lipid peroxidation—increased in IC- and DMSO-treated hearts but it was not modified by Isoesp treatment (Fig. 5b). 3-Nitrotyrosine immunoreactive bands (indicative of peroxynitrite production and protein nitration) were observed between 37 and 150 kDa (Fig. 5c). Analysis of all bands within this range revealed that in IC and DMSO hearts, 3-nitrotyrosine levels were 1.5-fold higher than those in NIC hearts. By contrast, in Isoesp-treated hearts, 3-nitrotyrosine levels were lower than those in IC or DMSO hearts. In Isoesp

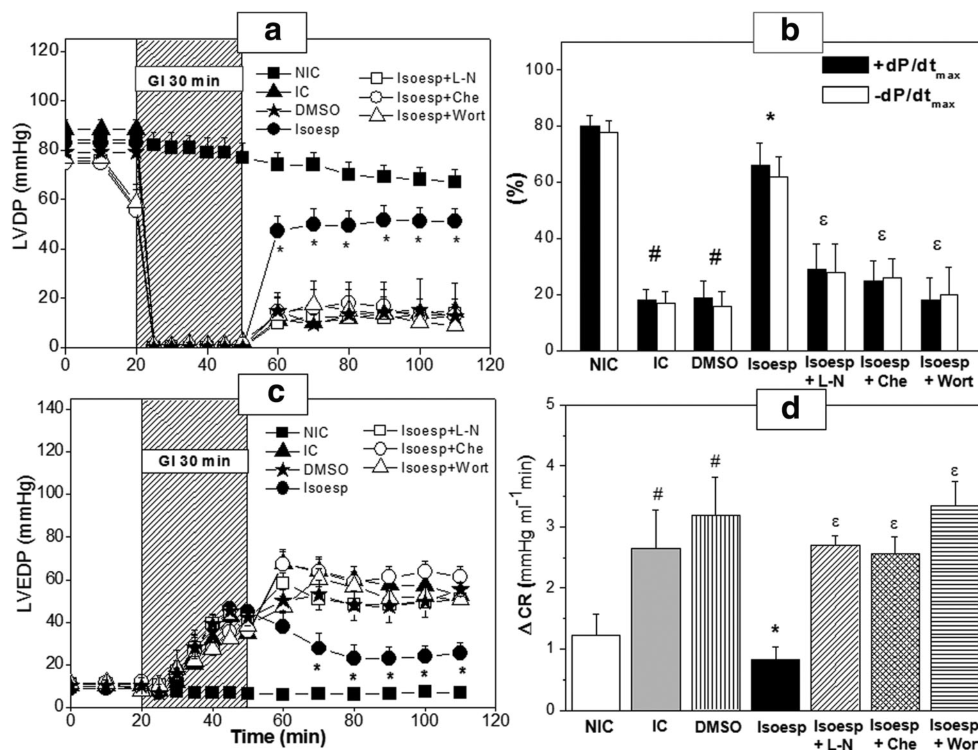

Fig. 4 Time course of left ventricular developed pressure (LVDP, **a**), values of $+dP/dt_{max}$ and $-dP/dt_{max}$ at the end of reperfusion (**b**), left ventricular end diastolic pressure (LVEDP, **c**) during ischemia-reperfusion, and changes of coronary resistance at 60 min of reperfusion (ΔCR, **d**), in NIC (n = 6), in IC (n = 8), and in hearts treated with DMSO (n = 6), Isoesp (n = 8), Isoesp + L-N (n = 6), Isoesp + Che (n = 6), and Isoesp + Wort (n = 6). Note that DMSO did not modify these parameters but Isoesp significantly improved the post-ischemic recovery of myocardial systolic and diastolic function and annulled the increase of CR detected in IC hearts. The NOS blockade with L-NAME, the PKC blockade with chelerythrine, and the PI3K/Akt blockade with wortmannin abolished the Isoesp-mediated effects. Statistical test: two-way ANOVA for **a** and **c** and one-way ANOVA for **b** and **d**, both followed by Bonferroni test. *$p < 0.05$ vs. IC or DMSO; $^{\#}p < 0.05$ vs. Isoesp

+ L-N, Isoesp + Che, and Isoesp + Wort, the 3-nitrotyrosine levels were comparable to those detected in IC or DMSO hearts.

Expression of P-Akt, P-eNOS, and P-PKCε

IC hearts showed a decrease of phospho-Akt (Fig. 6a), phospho-eNOS (Fig.6b), and phospho-PKCε (Fig. 6c) expression compared to NIC. Isoesp treatment reversed these effects showing Isoesp-treated hearts a significant increase of kinases and enzyme phosphorylation. These increases were modified when Isoesp was administered in the presence of NOS, PKC, and PI3K/Akt inhibitors. Thus, it was observed a decrease of P-Akt and P-eNOS expression in the Isoesp + Wort group, a decrease of P-eNOS and a small decrease of P-Akt in the Isoesp + L-N group and a decrease of P-PKCε and a slight but significant decay of P-Akt expression in the Isoesp + Che group.

Measurements in isolated mitochondria

Figure 7 shows values of parameters measured in mitochondrial samples. Light scattering decrease (LSD, Fig. 7a) after 100 μmol/L Ca^{2+} addition was 0.75 ± 0.03 a.u. This value was significantly attenuated in the presence of Isoesp (0.30 ± 0.04 a.u.). When cyclosporine A (a mPTP inhibitor) was added to mitochondrial sample, LSD was 0.12 ± 0.03 a.u. (data not shown) indicating the association between LSD and mPTP opening. Thus, minor changes of LSD are related to minor mPTP openings. Therefore, these data suggest that Isoesp is increasing the resistance of mPTP to open when Ca^{2+} is added. This resistance was also evident when calcium retention capacity (CRC, Fig.7b) was examined. CRC increases in the presence of Isoesp, indicating that this treatment enables mitochondria to accumulate and sustain Ca^{2+} before to promote the mPTP opening. Mitochondrial membrane potential (ΔΨm, Fig.7c) was not modified when Isoesp was added. Considering that potential plays a key role in a driving force for transport of ions, the maintenance of normal ΔΨm could indicate that a minimal amount of Ca^{2+} is entering the mitochondria.

Discussion

Our data show that Isoesp—a monoterpene isolated from *Oxandra* cf *xylopioides*—administered at the beginning of

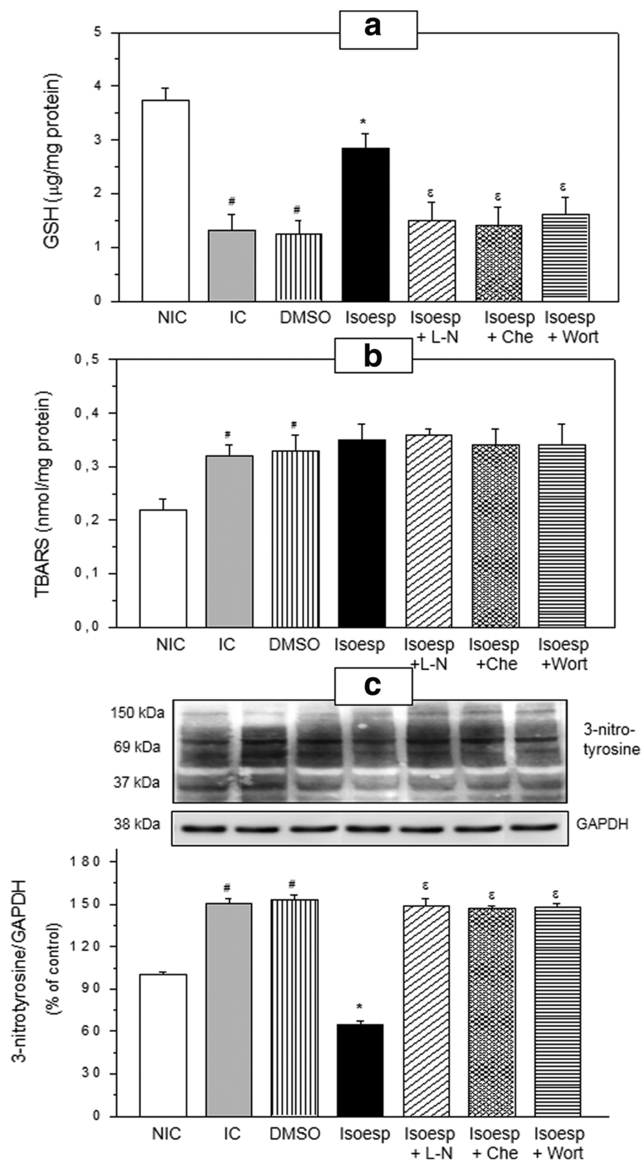

Fig. 5 Reduced glutathione content (GSH, **a**), thiobarbituric acid reactive substances (TBARS, **b**), and representative blot and summary of densitometry data of 3-nitrotyrosine (**c**) in NIC ($n = 5$), in IC ($n = 6$), and in hearts treated with DMSO ($n = 5$), Isoesp ($n = 6$), Isoesp + L-N ($n = 5$), Isoesp + Che ($n = 5$), and Isoesp + Wort ($n = 5$). GSH content decreased in IC and DMSO hearts, was partially preserved by Isoesp (~ 3 μg compared to ~ 1 μg in IC), and acquired similar values to IC hearts when Isoesp was administered in the presence of NOS or PKC or PI3K/Akt inhibitors. Lipid peroxidation—estimated by TBARS—increased in IC and was not modified by any treatment. The level of 3-nitrotyrosine increased in IC and DMSO hearts, decreased in Isoesp-treated hearts, and returned to values similar to those of IC or DMSO group when NOS, PKC, or PI3K/Akt were inhibited. Statistical test: one-way ANOVA with a Bonferroni multiple comparison post hoc test. $^*p < 0.05$ vs. IC or DMSO; $^\#p < 0.05$ vs. Isoesp

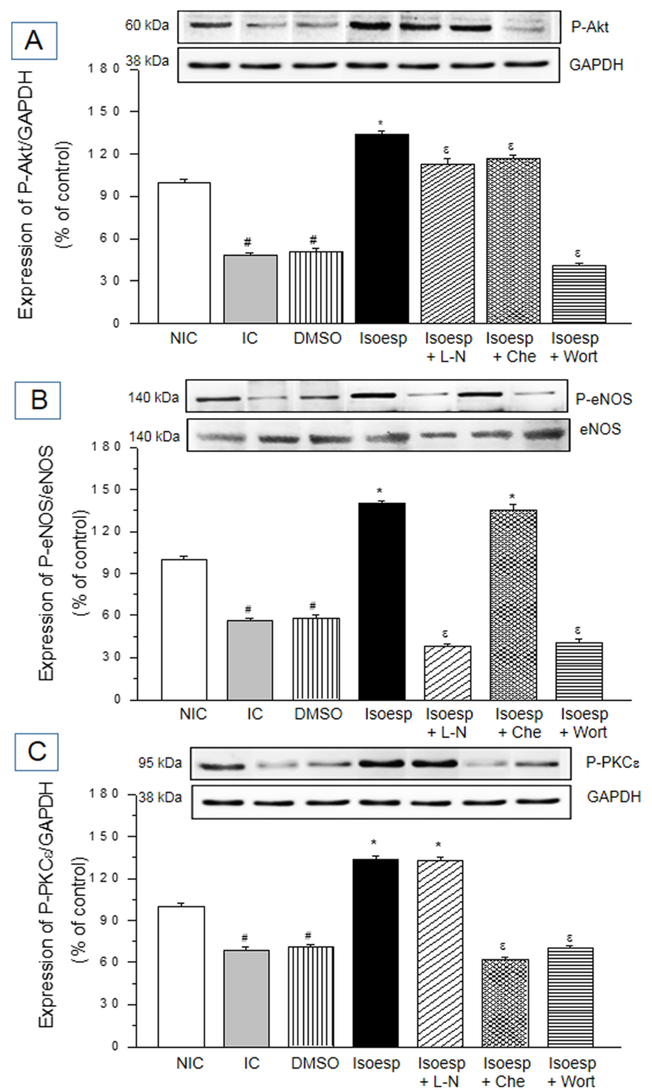

Fig. 6 Representative immunoblots of phosphorylated forms and summary of densitometry data of phospho-Akt (P-Akt, **a**), phospho-eNOS (P-eNOS, **b**), and phospho-PKCε (P-PKCε, **c**), in cardiac homogenate of NIC ($n = 5$), and IC ($n = 6$) and in hearts treated with DMSO ($n = 5$), Isoesp ($n = 6$), Isoesp + L-N ($n = 5$), Isoesp + Che ($n = 5$), and Isoesp + Wort ($n = 5$). After ischemia-reperfusion, the level of P-Akt, P-eNOS, and P-PKCε decreased and these values were not modified by DMSO. Isoesp increased the expression of these proteins. In Isoesp plus inhibitors (Isoesp + L-N, Isoesp + Che, and Isoesp + Wort), the expression of the respective proteins decreased, demonstrating the participation of Akt, eNOS, and PKCε in the effects of Isoesp. Statistical test: one-way ANOVA with a Bonferroni multiple comparison post hoc test. $^*p < 0.05$ vs. IC; $^\#p < 0.05$ vs. Isoesp

reperfusion, exerts beneficial actions during ischemia-reperfusion. Thus, this drug limits infarct size, improves cardiac function and coronary perfusion, and decreases oxidative stress. This study also suggests that Akt/eNOS/PKCε signaling pathways play a pivotal role in Isoesp-mediated cardioprotection.

An excessive ROS production, principally by mitochondria, and diminution of antioxidant reserve events occurring during reperfusion contribute to reperfusion injury (Lesnefsky et al. 2017; Murphy and Steenbergen, 2008; Raedschelders et al. 2012). On the other hand, an increase of NO—mediated by NOS activation—appears involved in cardioprotective mechanisms against ischemia-reperfusion injury (Bice et al. 2016). Interaction between superoxide anion

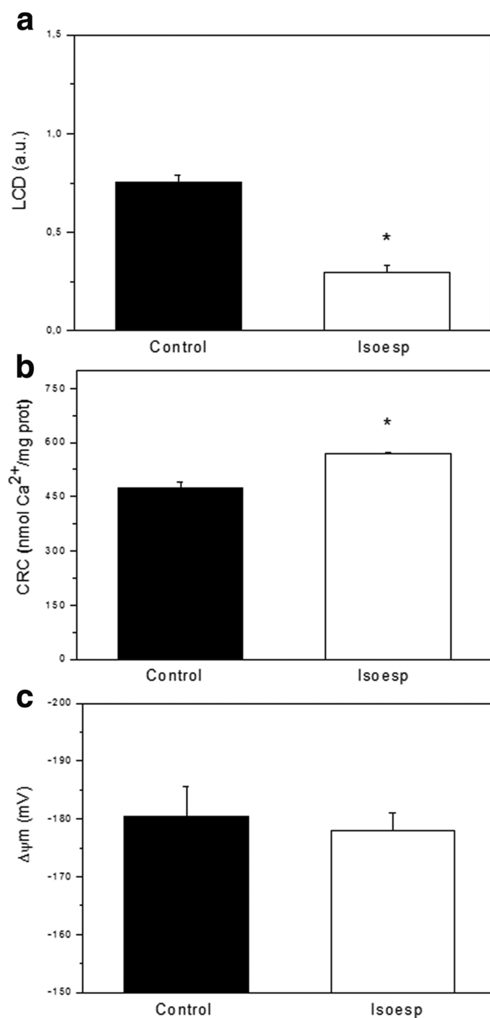

Fig. 7 Mean values of the light scattering decrease (LSD, **a**), calcium retention capacity (CRC, **b**), and mitochondrial membrane potential (ΔΨm, **c**) in mitochondrial suspensions in the absence (n = 5) and in the presence of Isoesp (n = 5). Isoesp significantly decreased the response of mitochondria to Ca^{2+} 100 μmol/L (evidenced by a diminution of LSD), increased CRC, but did not modify ΔΨm. Statistical test: unpaired Student's t test. *$p < 0.05$ vs. control

(a ROS) and NO generates peroxynitrite ($ONOO^-$) (Levrand et al. 2006; Ma et al. 1997). This specie is formed early during reperfusion and induces protein tyrosine residue nitration which contribute to development of cardiac dysfunction (Pacher et al. 2005). A decreased NO synthesis and increased ROS production are also mechanisms involved in the so-called no-reflow phenomenon (Kloner, 2011), responsible for abnormal coronary perfusion. Therefore, cell function and viability depend on NO/$ONOO^-$ balance. Our data show that Isoesp exerts anti-oxidative and anti-nitrosative actions and suggest that this compound is able to move NO/$ONOO^-$ balance towards NO. This effect could be implicated in Isoesp-mediated reduction of infarct size and improvement of irrigation. Taking into account that both effects are contributing to the improved clinical outcome of cardiovascular patients (Niccoli et al. 2019), Isoesp appears as a possible cardioprotective tool.

The mitochondria represents an essential target of cardioprotective strategies and preservation of mitochondrial function is a crucial event for reduction of ischemia-reperfusion injury. Oxidative stress and/or ATP depletion, followed by mitochondrial calcium loading to pathologically high levels, induce mPTP opening in the inner mitochondrial membrane. This event plays a fundamental role in cardiac reperfusion injury. When mPTP opens, mitochondrial swelling followed by dysfunction (diminished ATP production) occurs, and cell death rapidly ensues (Morciano et al. 2017). Thus, prevention of these mitochondrial alterations characterizes many cardioprotective interventions (Ong et al. 2015). In other words, mechanisms promoting mitochondrial homeostasis are essential for cell survival. In the present study, our data in isolated mitochondria demonstrate that Isoesp decreases mitochondrial calcium uptake and attenuates mPTP opening. Actions of NO at mitochondrial level, such as a diminution of nitration or an increase of nitrosylation of different structures or proteins associated to mPTP (Yang et al. 2012), could also be produced by Isoesp. Therefore, all these data seem to indicate that an attenuation of mitochondrial damage could be the mechanism responsible for Isoesp-mediated cardioprotection.

Now, the question is: What signaling pathways converge in mitochondria? It has been previously shown that cardioprotective effects induced by different maneuvers are mediated by PI3K/Akt and PKCε-dependent mechanisms (Matsui and Rosenzweig 2005; Inagaki et al. 2006; Heusch, 2015). Our data are clearly demonstrating that both kinases are involved in beneficial actions afforded by Isoesp treatment. To analyze possible interactions between PI3K/Akt and PKCε, we must consider previous data suggesting that Akt is upstream signaling of PKCε activation (Murphy and Steenbergen, 2008) or Akt is acting as a phosphorylation target for PKC (Kawakami et al. 2004). In our experimental conditions, PKC inhibition minimally modified P-Akt level indicating that PKC has a low contribution to Akt phosphorylation. Differently, Akt blockade was accompanied by an important decrease of PKCε phosphorylation pointing out that Akt appears as a possible way of PKC activation. On the other hand, and taking into account that Akt can activate eNOS (Kawasaki et al. 2003), simultaneous increase of P-eNOS and P-Akt detected in Isoesp-treated hearts indicate that Akt/eNOS is the possible pathway activated by that monoterpene. Additionally, eNOS is a possible target of PKCε (Zhang et al. 2005) and also NO can activate PKCε (Ping et al. 1999). In this study, P-eNOS content did not change when PKC was inhibited indicating that eNOS is not activated by PKC. Furthermore, the possibility of NO-mediated PKC activation was ruled out because when NOS was inhibited, PKCε phosphorylation was not modified.

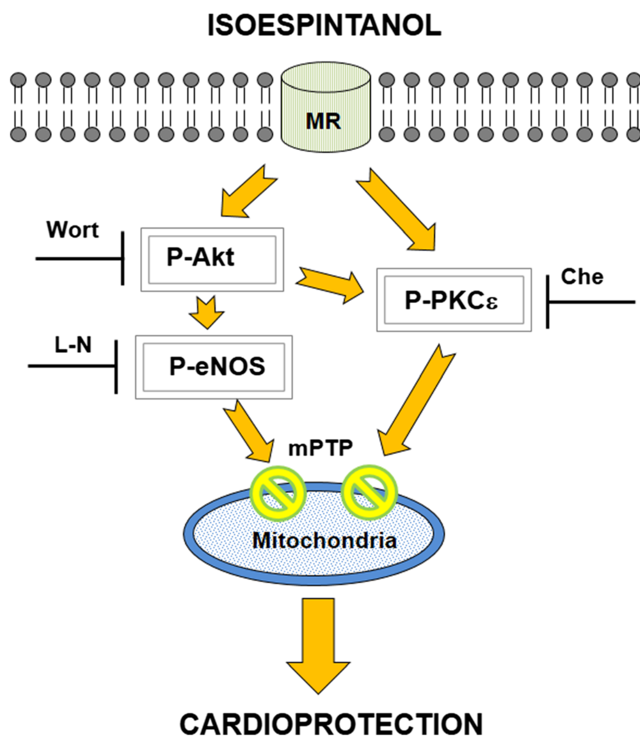

Fig. 8 Mechanisms involved in the isoespintanol-mediated cardioprotection. The impact of isoespintanol on membrane receptors (MR) activates Akt/eNOS/PKCε-mediated intracellular pathways that produce an attenuation of mPTP opening event associated to the cardioprotection

Conclusions

This study demonstrate that isoespintanol administered only at reperfusion is cardioprotective. Thus, in a global ischemia model, this drug reduces cell death, contractile dysfunction, and impaired irrigation. Also, our data show that Akt/eNOS- and PKCε-dependent pathways targeting mitochondria and improving its performance are the most important mechanisms responsible of Isoesp-mediated protective actions (Fig. 8). Although the results of this investigation are promisorious, further studies to demonstrate their "in vivo" efficacy and safety are necessary.

Author contributions L.G.A. performed the western blots; A.C.P. performed the mitochondrial experiments; J.F. performed the isolated hearts experiments; B.J. isolated and provided the drug; G.Sch. contributed to the conception of the study; S.M. wrote the manuscript. All the authors contributed to the analysis and interpretation of data, critically reviewed, and approved the final draft.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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