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Phenolic Metabolites Modulate Cardiomyocyte Beating in Response to Isoproterenol

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Abstract

Cardiovascular disease (CVD) is a public health concern, and the third cause of death worldwide. Several epidemiological studies and experimental approaches have demonstrated that consumption of polyphenol-enriched fruits and vegetables can promote cardioprotection. Thus, diet plays a key role in CVD development and/or prevention. Physiological β-adrenergic stimulation promotes beneficial inotropic effects by increasing heart rate, contractility and relaxation speed of cardiomyocytes. Nevertheless, chronic activation of β-adrenergic receptors can cause arrhythmias, oxidative stress and cell death. Herein the cardioprotective effect of human metabolites derived from polyphenols present in berries was assessed in cardiomyocytes, in response to chronic β-adrenergic stimulation, to disclose some of the underlying molecular mechanisms. Ventricular cardiomyocytes derived from neonate rats were treated with three human bioavailable phenolic metabolites found in circulating human plasma, following berries' ingestion (catechol-*O*-sulphate, pyrogallol-*O*-sulphate, and 1-methylpyrogallol-*O*-sulphate). The experimental conditions mimic the physiological concentrations and circulating time of these metabolites in the human plasma (2 h). Cardiomyocytes were then challenged with the β-adrenergic agonist isoproterenol (ISO) for 24 h. The presence of phenolic metabolites limited ISO-induced mitochondrial oxidative stress. Likewise, phenolic metabolites increased cell beating rate and synchronized cardiomyocyte beating population, following prolonged β-adrenergic receptor activation. Finally, phenolic metabolites also prevented ISO-increased activation of PKA–cAMP pathway, modulating Ca^{2+} signalling and rescuing cells from an arrhythmogenic Ca^{2+} transients' phenotype. Unexpected cardioprotective properties of the recently identified human-circulating berry-derived polyphenol metabolites were identified. These metabolites modulate cardiomyocyte beating and Ca^{2+} transients following β-adrenergic prolonged stimulation.

Keywords Cardiomyocytes · β-Adrenergic receptors · Isoproterenol · Human bioavailable phenolic metabolites · Polyphenols

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Abbreviations

β-AR	β-Adrenergic receptors
BDP	Berry-derived polyphenols
CaMKII	Calcium calmodulin-dependent kinase II
cAMP	Cyclic adenosine monophosphate
CVD	Cardiovascular diseases
ISO	Isoproterenol
PKA	cAMP-dependent protein kinase A
ROS	Reactive oxidative species
RyR	Ryanodine receptors
SR	Sarcoplasmic reticulum
SERCA	Sarcoplasmic reticulum calcium-ATPase
GPCRs	G protein-coupled receptors

Introduction

Cardiovascular diseases (CVD) are the third cause of death worldwide (http://www.euro.who.int). Because of the increased life expectancy, the occurrence of CVD is growing dramatically. Moreover, sedentary lifestyle and unhealthy diet strongly contribute for CVD rising incidence and progression (http://www.euro.who.int). CVD present multifactorial aetiologies, affecting heart and vessels, and comprise hypertension, atherosclerosis, myocardial infarction and heart failure. In response to stressful conditions, the heart can undergo several compensatory processes to normalize cardiovascular function. Thus, activation of the sympathetic nervous system in the heart, particularly via β-adrenergic receptors (β-AR), is crucial for the acute response and improvement of cardiac output under stressful situations [1]. Circulating catecholamines or other β-AR agonists [such as isoproterenol (ISO)] activates β-AR, which in turn, increases cardiomyocytes' contractility and relaxation speed, and heart rate. Stimulation of β-AR promotes transmembrane adenylyl cyclase activity and production of the second messenger cyclic adenosine monophosphate (cAMP), which activates cAMP-dependent protein kinase A (PKA). PKA mediates the phosphorylation of key proteins involved in Ca^{2+} excitation–contraction coupling and myofibrillar proteins [2–4]. Thus, β-AR stimulation increases cytoplasmic Ca^{2+} levels, due to Ca^{2+} entry into the cytoplasm via phosphorylated L-type channels and due to Ca^{2+} release from the sarcoplasmic reticulum (SR) via phosphorylated ryanodine receptors (RyR) [2, 5, 6]. Furthermore, β-AR stimulation also enhances Ca^{2+} uptake from the cytoplasm to the SR via sarcoplasmic reticulum calcium-ATPase (SERCA) [2, 5, 6]. Likewise, enhancement of cytosolic Ca^{2+} levels also contributes to the activation of the Ca^{2+} calmodulin-dependent kinase II (CaMKII), leading to the phosphorylation of key proteins related to Ca^{2+} signalling and excitation–contraction coupling [2–6]. By facilitating the release and uptake of Ca^{2+} from and to the sarcoplasmic reticulum, activation of β-AR promotes the increase of Ca^{2+} transients, inducing cardiomyocytes' contraction and relaxation speed and increasing cell beating rate [2, 3, 5, 6]. Nevertheless, sustained adrenergic stimulation can promote decompensation, and the development of heart failure and cardiac arrhythmias [4, 7]. In fact, continuous activation of β-AR is associated with increasing oxidative stress, deleterious modulation on Ca^{2+} signalling and generation of arrhythmogenic Ca^{2+} waves [7–10]. Furthermore, treatment with ISO for 48 h induced deleterious adrenergic stimulation and triggered cell death in a mitochondria membrane permeabilization-dependent manner, in cardiomyoblasts (H9C2 cell line) [10, 11].

Numerous epidemiological studies have correlated high consumption of vegetables and fruits to a reduction of CVD. High levels of polyphenols are described as crucial components for the cardioprotective effects of vegetables and fruit-enriched diets [12–15]. Polyphenols are phytochemicals found in plants and they are characterized by the presence of one or more phenol structures. Experimental models and clinical studies showed that polyphenol-enriched products may activate antioxidant defences, reduce inflammation, limit endothelial dysfunction, promote vasodilation and decrease cardiac cell death [14–17]. Moreover, some polyphenols can also limit damage caused by cardiac remodelling [16, 18]. Since polyphenol compounds are identified as xenobiotics by other organisms, they are extensively metabolized, which alters their chemical features, their bioavailability and, consequently, their effects [19]. The majority of in vitro studies have identified the cardioprotective role of polyphenols by directly testing them on cell culture models, without considering their metabolization or bioavailability [20]. Recently, Pimpão et al. performed a cross-over human intervention study using a specific berry-mixed *purée* containing commercial blueberries, blackberries and raspberries, wild Portuguese crowberry and strawberry tree fruit. This study allowed the identification of several phenolic metabolites present in the plasma and urine of healthy volunteers, as well as their circulating time and concentration [21, 22]. Thus, the use of human phenolic metabolites on cardiomyocytes' cultures to disclose the molecular mechanisms underlying the cardioprotective effects of polyphenols is a unique and physiological relevant experimental approach.

Herein it is hypothesized that dietary polyphenols via their bioavailable metabolites may be cardioprotective by modulating cardiomyocytes' function and fate. Thus, the three most abundant metabolites identified in the plasma of human volunteers (catechol-*O*-sulphate, pyrogallol-*O*-sulphate or 1-methylpyrogallol-*O*-sulphate) were applied to rat neonatal cardiomyocytes and differentiated cardiomyoblast H9c2 cells. Cells were exposed to these compounds at their physiological concentration and for a time period mimicking their circulating time in human plasma [21, 22]. Cardiomyocytes were challenged with the β-AR agonist ISO, to mimic deleterious β-AR stimulation. While the three metabolites were not able to prevent ISO-induced cell death, they decreased mitochondrial ROS generation and improved cell beating. Likewise, these metabolites also prevented ISO-increased activation of PKA–cAMP pathway, modulating Ca^{2+} transients and limiting arrhythmogenic Ca^{2+} transients' phenotype.

Materials and Methods

Animals

Wistar rat pups obtained from NOVA Medical School animal facility were used for isolating neonatal cardiomyocytes. All animals were specific-pathogen-free according to FELASA recommendations [23]. All applicable institutional and governmental regulations concerning the ethical use of animals were followed, according to the NIH Principles of Laboratory Animal Care (NIH Publication 85-23, revised 1985), the European guidelines for the protection of animals used for scientific purposes (European Union Directive 2010/63/EU) and the Portuguese Law no. 113/2013, and approved by *Direção Geral de Alimentação e Veterinária*.

H9c2 Cell Culture and Differentiation

Cardiomyoblast H9c2 cell line was cultured in plating medium 89% (v/v) Dulbecco's modified Eagle's medium (DMEM) medium supplemented with 4.5 g/L of glucose, 0.11 g/L of sodium pyruvate and L-glutamine (Sigma), 10% (v/v) Foetal Bovine Serum (FBS, Sigma), and 1% (v/v) 50 U/mL of penicillin and 50 µg/mL of streptomycin (Sigma). Cells were maintained at 37 °C and 5% CO_2, in plating medium, changed every 2–3 days. Once cells reached 70–80% of confluence, H9c2 cells were subcultured to avoid loss of differentiation ability. 24 h after subculture H9C2, cell differentiation to cardiac muscle-like cells was conducted by decreasing the media serum to 1% (v/v) Horse Serum (Gibco) and by adding 1 µM all-trans retinoic acid (Sigma). Differentiation medium was prepared daily, and cell medium was changed daily for 5 days. After the 5 days differentiation protocol, cells were maintained and manipulated in differentiation medium.

Neonatal Cardiomyocyte Isolation

Ventricular neonatal cardiomyocytes were isolated from 2- to 3-day old Wistar rat pups, following a protocol adapted from Louch et al. [24]. Briefly, all animals were rapidly euthanized by decapitation and their hearts were removed. Ventricles were minced in ADS buffer (0.12 M NaCl, 1.36 mM NaH_2PO_2, 5.5 mM glucose, 5.37 mM KCl, 0.406 mM $MgSO_4$, 18 mM Hepes) and enzymatically digested with 30 mg Pancreatin (Sigma) and 26 mg Collagenase (Roche), for 20-min cycles, at 37 °C. The samples were then centrifuged for 5 min. Collected fractions were kept at 37 °C and 5% CO_2, resuspended in New Born Calf Serum (Gibco). At the end, all fractions were pooled, resuspended in ADS buffer and added to a Percoll gradient (Sigma). After centrifugation for 30 min at 2095×*g*, purified cardiomyocytes were plated in plating medium 68% (v/v) DMEM GlutaMax supplemented with 1 g/L glucose and 0.11 g/L sodium pyruvate (Gibco), 17% (v/v) Medium 199 supplemented with 2.2 g/L sodium bicarbonate and 0.1 g/L L-glutamine (Sigma), 10% (v/v) Horse Serum (Gibco), 5% (v/v) New Born Calf Serum (Gibco), 1% (v/v) L-glutamine (Gibco) at a concentration of 7.4×10^4 cells/mL. Plastic plates were coated with 0.1% (w/v) gelatine and glass plates were coated with 100 µg/mL laminin. Cells were maintained, at 37 °C and 5% CO_2, in plating medium, changed every 3 days, until desirable confluence was reached (around 7 days) [24].

Phenolic Metabolites Pre-treatment and Isoproterenol Exposure

Phenolic metabolites were chemically synthesized as previously described [22]. The following experimental conditions are based on the concentration and circulating time of the metabolites in the human plasma, found by Pimpão et al. [22].

H9c2-differentiated cells and ventricular neonatal cardiomyocytes were pre-treated, for 2 h at 37 °C and 5% CO_2, with the different metabolites (Supplementary Fig. 1), at their circulating concentrations (catechol-*O*-sulphate: 12 µM; pyrogallol-*O*-sulphate: 6 µM; and 1-methylpyrogallol-*O*-sulphate: 3 µM). After washing out the metabolites, cells were then exposed to 200 µM ISO (Sigma) in fresh plating medium for 24 h or 48 h. Control cells were incubated for the same period, at 37 °C and 5% CO_2, with new medium as the ones exposed to the phenolic metabolites and/or ISO. Control cells were also subject to the same washout process as cells exposed to phenolic metabolites, after 2 h. A cytotoxicity assay was previously performed for these phenolic metabolites in cardiomyocytes. They do not present any toxic effect.

Cell Viability

H9c2 cell viability was assessed by propidium iodide (PI; 1 µg/mL) (Invitrogen, UK). Flow cytometry (Canto II device) was used to analyse cell death-associated parameters. This cytometer contains a FL3 red fluorescence channel for PI detection at 650 nm. The acquisition was done with Diva software and data analysis was performed with Flowing software. Neonatal rat cardiomyocyte viability was quantified by Trypan blue dye and cell counting, using a Zeiss Axiovert 40 widefield microscope. Both assays are based on the plasma membrane integrity. At least four different biological replicates, derived from different heart pools, were used.

Mitochondrial Reactive Oxygen Species (ROS) Evaluation

After pre-treatment with phenolic metabolites for 2 h, and exposure to ISO, for 24 h, cellular medium was discarded, and ventricular neonatal cardiomyocytes were washed with phosphate buffered saline pH 7.2. Cells were incubated with 1 µM MitoSox (Invitrogen), for 30 min, at 37 °C, in the dark. ROS levels were measured using a Tecan Infinite F200 PRO plate reader. At least four different biological replicates were used, each derived from a different heart pool.

Cell Beating Measurement

Ventricular neonatal cardiomyocytes' cell beating was recorded using a Nikon Eclipse Ti-U inverted bright field light microscope (×10/0.25 NA dry objective lens) at room temperature (RT) (25 °C). Images were recorded by High Speed FASTCAM MC2 camera (Photron Europe, Limited) and Photon FASTCAM Viewer software, with a speed of 60 frames/s (total of 1500 frames per microscopic field). By the end of an experimental set, 20 microscopic fields (20 conditions) were recorded. Temperature is important when assessing cardiomyocytes' beating. To avoid any artefact due to non-optimal temperature and pH (25 °C, atmospheric pressure), all experimental conditions were recorded in a serial manner. No significant differences were detected between the first and the last replicates for each condition. Using the recorded videos, cell beating was evaluated. From each microscopic field, different cell clusters were studied. And from each cell cluster, individual cells were analysed using Fiji software (total of 20 cell per condition) [25]. The background of cells' surface shape was removed, and images were converted to grayscale (16 bits per sample pixel). Cardiomyocytes' beating was evaluated by measuring light intensity variation through time (1500 frames). The frequency of light alteration was considered as the frequency of cell beating (beats/min). At least, three different experimental sets were performed, using cells from different heart pools for each experiment.

Western Blot

Samples were collected using RIPA lysis buffer (5 mM Tris–HCL, 15 mM NaCl, 0.01% SDS, 0.1% Sodium deoxycholate, 0.1% Triton X-100) with 1% (v/v) Protease inhibitor. Protein concentration was quantified using Pierce BCA Protein Assay Kit. Protein extracts (30 µg per sample) were subjected to a 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis for 1 h at 150 V and electrically transferred for 1 h and 15 min to a PVDF membrane. Membranes were blocked with 5% (w/v) milk, 0.01% (v/v) Tween20 Tris-buffered saline, for 1 h at RT. Membranes were incubated with PhosphoPKA [26, 27] (1:1000; Cell Signalling #4781), PhosphoCaMKII [28–30] (1:1000; Invitrogen MA1-047), Total PKA (1:1000; Cell Signalling #4782) or Total CaMKII (1:200; Santa Cruz Biotechnology sc-9035) in 5% (w/v) bovine serum albumin (BSA), 0.01% (v/v) Tween20 Tris-buffered saline overnight at 4 °C. Incubation with ECL-anti-mouse IgG horseradish peroxidase-linked whole antibody (1:5000; GE Healthcare NA931) or ECL-anti-rabbit IgG horseradish peroxidase-linked whole antibody (1:5000; GE Healthcare NA934) was performed for 1 h at RT. Proteins were detected in a ChemiDoc Touch Imaging System (Bio-Rad) and images were analysed using Image Lab (BioRad) and Fiji software [25]. Four different biological replicates were used and each derived from a different heart pool.

Analysis of Calcium Transients by Confocal Microscopy

Ventricular neonatal cardiomyocytes were incubated with 10 µM of the high-affinity Ca^{2+} indicator Fluo-4 AM (Invitrogen F10471), at 37 °C in the dark, for 30 min in Tyrode solution (0.14 M NaCl, 5.4 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 10 mM glucose and 10 mM Hepes pH 7.4) [31–33]. Cell were washed with Tyrode solution and stayed 20 min in the dark with Tyrode solution, prior to image acquiring. For each microscopic field, a total of 800 frames (6 frames/s) were recorded during 2 min, using a Andor Spinning disk confocal inverted microscope (×20) with an enclosed incubator with controlled temperature at 37 °C. Fluo-4 AM was excited with an OPSL CW 488-nm laser and fluorescence was measured with a 488LP filter (500–1100 nm). Changes of cytosolic Ca^{2+} are presented as normalized fluorescence (F/F_0), where F stands for fluorescence intensity at time t; F_0 is the mean baseline fluorescence, calculated from frames recorded between beats. Ca^{2+} transient amplitude was calculated as the mean of the fluorescence maximum at each Ca^{2+} transient. At least, three different biological replicates, each from a different pool of hearts, were recorded. A total of ten cells were evaluated from different clusters from each condition. Although there was no CO_2 controlled chamber in the confocal microscope, during the assay, there were not any alterations to register on Tyrode buffer's pH.

Statistics

Data are presented as mean ± standard deviation. Reported results were statistically evaluated using Sigma Plot 11 software (Systat Software Inc.) and GraphPad Prism (GraphPad software Inc.). Data were analysed by two-way analysis of variance (two-way ANOVA) on ranks and compared by Tukey's multiple comparison test with a confidence levels of 95%. Test results were considered significant at $P < 0.05$.

Results

Phenolic Metabolites Do Not Inhibit Cell Death in Cardiomyocytes Challenged with ISO

Several polyphenols have been described to inhibit cell death in independent studies [14, 16, 17]. Thus, cardiomyocyte culture was treated with human bioavailable phenolic metabolites to address their potential cardioprotective role. In fact, differentiated cardiomyoblast H9c2 cells and primary cultures of ventricular neonatal cardiomyocytes were pre-treated with three human bioavailable metabolites: catechol-*O*-sulphate, pyrogallol-*O*-sulphate or 1-methylpyrogallol-*O*-sulphate (Supplementary Fig. 1) for 2 h and washed out. The used metabolites' concentrations are the concentrations found in human plasma by Pimpão et al. [22]. Also, their time of exposure to cardiomyocytes mimics the time period that these metabolites are in circulation in human plasma and can contact myocardium tissue [22]. Since ISO is a widely used cardio-specific cell death inducer, following phenolic metabolite's treatment, differentiated H9c2 cells were exposed to 200 µM ISO for 48 h (Supplementary Fig. 2) and neonatal cardiomyocytes were exposed to ISO for 24 h (Fig. 1a) and 48 h (Fig. 1b). ISO treatment for 24 h did not induce cardiomyocytes' cell death (Fig. 1a), but at 48 h, ISO induced cell death in differentiated H9c2 cells (Supplementary Fig. 2) and neonatal cardiomyocytes (Fig. 1b). These results are in accordance with Branco et al.'s previous work [10, 11]. Still, pre-treatment with catechol-*O*-sulphate, pyrogallol-*O*-sulphate or 1-methylpyrogallol-*O*-sulphate did not protect cardiomyocytes against cell death, following 48 h of ISO treatment (Supplementary Fig. 2 and Fig. 1b). In conclusion, unlike other polyphenol compounds, none of the metabolites conferred cytoprotection by a direct effect on cell death inhibition.

Phenolic Metabolites Modulate Cardiomyocyte Cell Beating and Its Synchronization

Cardiomyocyte beating was measured in the presence of phenolic metabolites to assess their role on cardiomyocytes' function. The beating rate of cardiomyocytes is greatly dependent on cell-to-cell interaction and electrical coupling. Therefore, it depends on cell plating density and on the formation of cell clusters. From each recorded microscopic field, several individual cells from different cell clusters were analysed. A total of 20 cells from different clusters were evaluated, per condition. Each time a cell beats there is an alteration of the light intensity recorded by the microscope. Thus, cardiomyocytes' beating frequency was considered equal to the light variation frequency through time.

Continued ISO treatment for 24 h decreased the average beating rate of cardiomyocytes (Fig. 2). Likewise, within the ISO-treated cardiomyocyte population, there is a more asynchronized pattern of cell beating, which is observed by the larger distribution of cell beating population (Fig. 2). These results are in accordance with the asynchronous behaviour of cardiomyocytes described in the prolonged exposure to ISO [8, 9]. When cardiomyocytes were pre-treated for 2 h with the phenolic metabolites and then exposed to ISO, there was a reversion of ISO deleterious effects. Phenolic metabolites increased cell beating rate to levels closer to control cells and cell beating distribution was narrower (Fig. 2a). Thus, in the presence of ISO-induced chronic stress, pre-treating cardiomyocytes with phenolic metabolites modulated their beating by

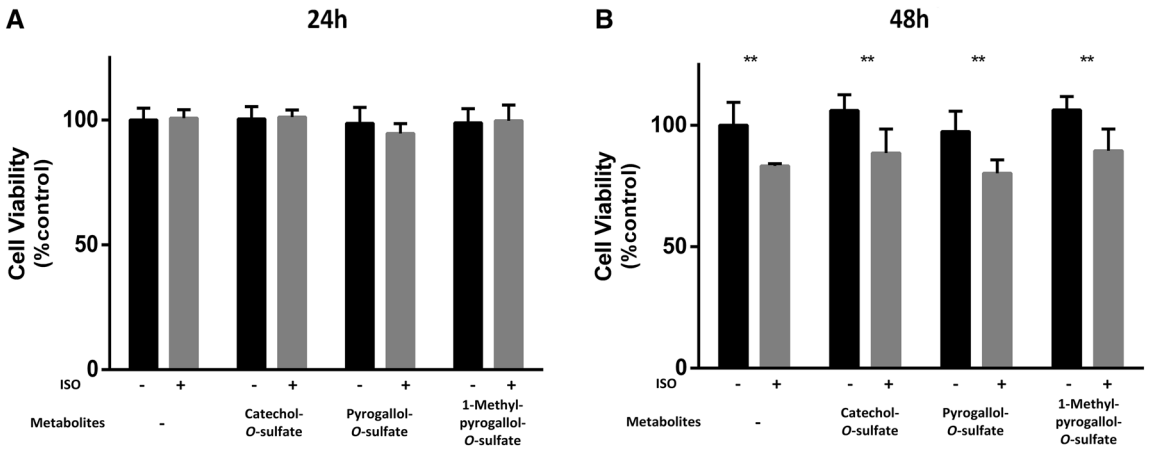

Fig. 1 Cell viability quantification. Cell Viability of neonatal rat cardiomyocytes, treated with phenolic metabolites for 2 h and exposed to 200 µM ISO for 24 h (**a**) or 48 h (**b**). Data are mean ± SD, from three independent experiments (in each condition, $n = 4$ per independent experiment). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (*ISO* isoproterenol)

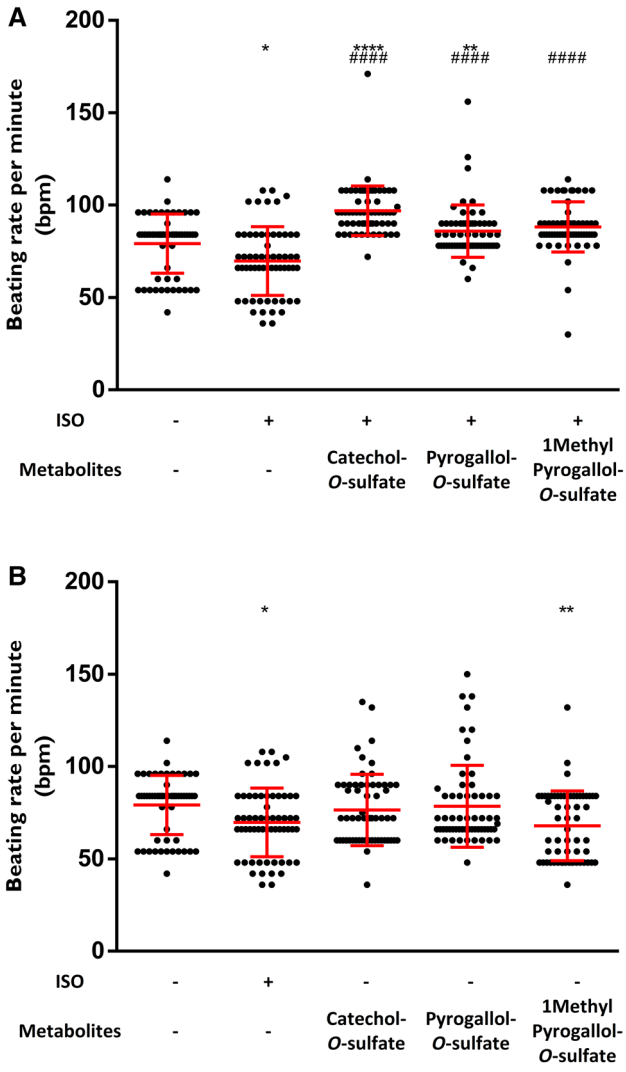

Fig. 2 Cardiomyocytes' cell beating. Neonatal rat cardiomyocytes' cell beating measurement, after 2 h treatment with phenolic metabolites and exposure (**a**) or not (**b**) to 200 µM ISO for 24 h. Filled circle represents a beating cell and the red bar stands for the mean beating rate per minute. Data are mean ± SD from at least three independent experiences. In each condition, beating rates of 20 cells were measured per independent experiment. $^{*}P<0.05$, $^{**}P<0.01$, $^{****}P<0.0001$ versus control. $^{\#\#\#\#}P<0.0001$ versus ISO (*ISO* isoproterenol)

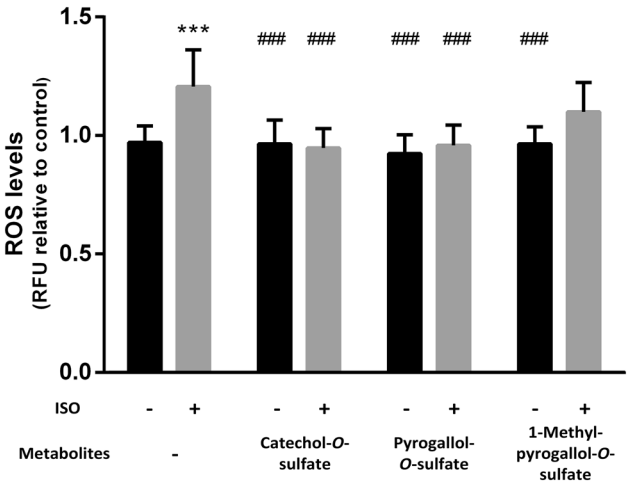

Fig. 3 Mitochondrial ROS quantification in neonatal rat cardiomyocytes. ROS was evaluated after a 2-h pre-treatment with the different phenolic metabolites and treatment for 24 h with 200 µM ISO. Data are mean ± SD, from three independent experiments (in each condition, $n=4$ per independent experiment). Superoxide levels were detected by MitoSox Red. $^{***}P<0.001$ versus control. $^{\#\#\#}P<0.001$ versus ISO (*ISO* isoproterenol)

increasing cell beating rate and/or by synchronizing spontaneous cell beating. Interestingly, exposure to phenolic metabolites alone also modulates cardiomyocyte beating. In the presence of the three metabolites, cardiomyocytes' beating distribution is larger, similarly to ISO treatment (Fig. 2b). Furthermore, 1-methylpyrogallol-*O*-sulphate decreased cardiomyocytes' beating to levels close to ISO-treated cells (Fig. 2b). These results suggested that these phenolic metabolites may directly modulate cell beating and eventually correct spontaneous beating defects.

Phenolic Metabolites Decrease Mitochondrial ROS Production in Cardiomyocytes Exposed to ISO

To further disclose whether these metabolites can directly target cardiomyocytes and promote other beneficial effects, ROS production was measured. Neonatal cardiomyocytes were exposed to ISO for 24 h, which is enough to promote cellular stress and mitochondrial ROS production (anion superoxide), without triggering cell death (Figs. 1, 3) [7–10]. Mitochondrial anion superoxide levels were quantified by MitoSox dye following ISO treatment in the absence or presence of phenolic metabolites' pre-treatment (Fig. 3). Pre-treatment with catechol-*O*-sulphate, pyrogallol-*O*-sulphate or 1-methylpyrogallol-*O*-sulphate significantly decreased the levels of anion superoxide accumulated following ISO exposure. These results suggest that phenolic metabolites' pre-treatment limits ISO-induced oxidative stress, a key element of the deleterious effect of prolonged ISO stimulation.

Phenolic Metabolites Modulate PKA/cAMP Pathway

A tight control of Ca^{2+} signalling is crucial for cell beating modulation. Stimulation of β-AR induces the activation of PKA/cAMP pathway, promoting the occurrence of Ca^{2+} transients and stimulating cellular contraction [2–6]. Moreover, Ca^{2+} activates CaMKII kinase, which also modulates cardiomyocyte beating. Our data suggest that the tested phenolic metabolites improve cardiomyocytes' beating rate in response to ISO (Fig. 2). Therefore, it can be hypothesized that these metabolites may regulate cardiomyocyte

beating via PKA/cAMP pathway and CaMKII kinase. Thus, the phosphorylation state of these kinases was evaluated for addressing PKA and CaMKII activation in the presence of the phenolic metabolites. Cardiomyocytes exposed to ISO have increased their PKA phosphorylation levels (Fig. 4a). Pre-treatment with pyrogallol-*O*-sulphate significantly decreased phosphorylation levels of PKA under both conditions: control and adrenergic stress. However, catechol-*O*-sulphate exposure significantly decreases PKA activation only following ISO treatment (Fig. 4a). 1-methylpyrogallol-*O*-sulphate also seems to decrease PKA phosphorylation in response to prolonged ISO treatment, but in a non-statistical significant manner (Fig. 4a). Likewise, the three phenolic metabolites significantly limited phosphorylation and activation of CaMKII in response to ISO treatment (Fig. 4b). However, there is also a significant increase in CaMKII activation due to phenolic metabolites presence, without ISO stimulation. These data indicate that these metabolites might directly activate CaMKII in cardiomyocytes, possibly in a PKA-independent way. This might contribute to the asynchrony cell beating phenotype observed in Fig. 2b. Likewise, these results suggest that when cells are exposed to ISO, these phenolic metabolites can directly modulate Ca^{2+} signalling pathways, decreasing the activation of PKA and CaMKII, which may contribute for the modulation of cell beating to a pattern closer to control cells. In addition, these three compounds are also capable of altering CaMKII activation levels without the presence of ISO (Fig. 4b), suggesting they can directly modify cell beating, by modulating the Ca^{2+} signalling pathways.

Phenolic Metabolites Modulate Ca^{2+} Transients and Their Amplitude

Normal beating rate, contractility and relaxation of cardiomyocytes are highly dependent on Ca^{2+} transients' amplitude and frequency [2, 3, 5, 6]. It has been described that continued treatment with ISO generates arrhythmogenic Ca^{2+} transients [8, 9]. In addition, our previous data suggest phenolic metabolites modulate cardiomyocytes' beating rate and frequency, as well as Ca^{2+} signalling via modulation of PKA and CaMKII activation, after prolonged ISO treatment (Figs. 2a, 4). Thus, to confirm whether Ca^{2+} transients are affected by these phenolic metabolites, evaluation of Ca^{2+} transients was addressed by confocal microscopy. Cardiomyocytes were treated with phenolic metabolites for 2 h followed by 24-h exposure to ISO to promote chronic stress; then Ca^{2+} transients were measured by fluorescence. Fluo4 fluorescence was evaluated using several cells from different cell clusters, per condition.

Non-treated cardiomyocytes present constant Ca^{2+} transients throughout the period of acquisition (Fig. 5a top left panel). In contrast, prolonged exposure to ISO generates unsteady Ca^{2+} transients, with several pauses between them (Fig. 5a top right panel). Moreover, cardiomyocytes exposed to ISO present transients with significantly higher amplitude

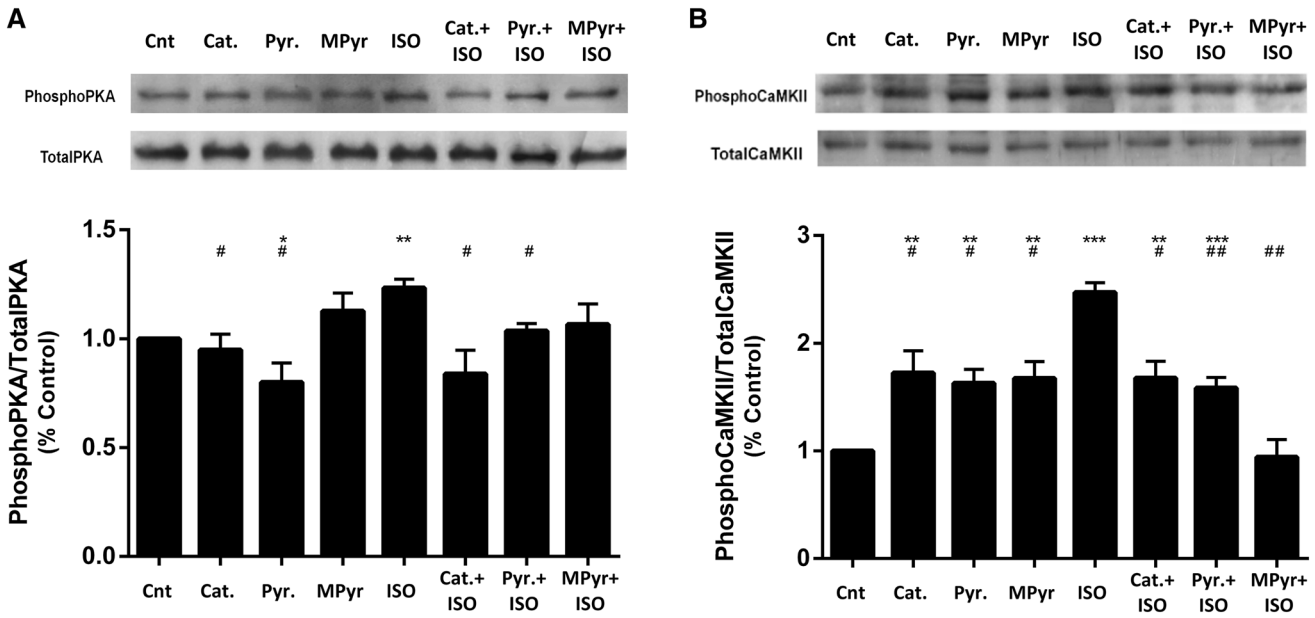

Fig. 4 Ca^{2+} signalling. Activation of Ca^{2+} signalling in neonatal cardiomyocytes, after exposure to phenolic compounds and ISO: representative image and quantitative analysis of PKA activation (**a**) in cardiomyocytes; and representative image and quantitative analysis of CaMKII activation (**b**) in neonatal cardiomyocytes. Data are mean ± SD from three independent experiences. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ versus Control. $^{\#}P<0.05$, $^{\#\#}P<0.01$ versus ISO (*Cnt* control, *Cat* catechol-*O*-sulphate, *Pyr* pyrogallol-*O*-sulphate, *MPyr* 1-methylpyrogallol-*O*-sulphate, *ISO* isoproterenol)

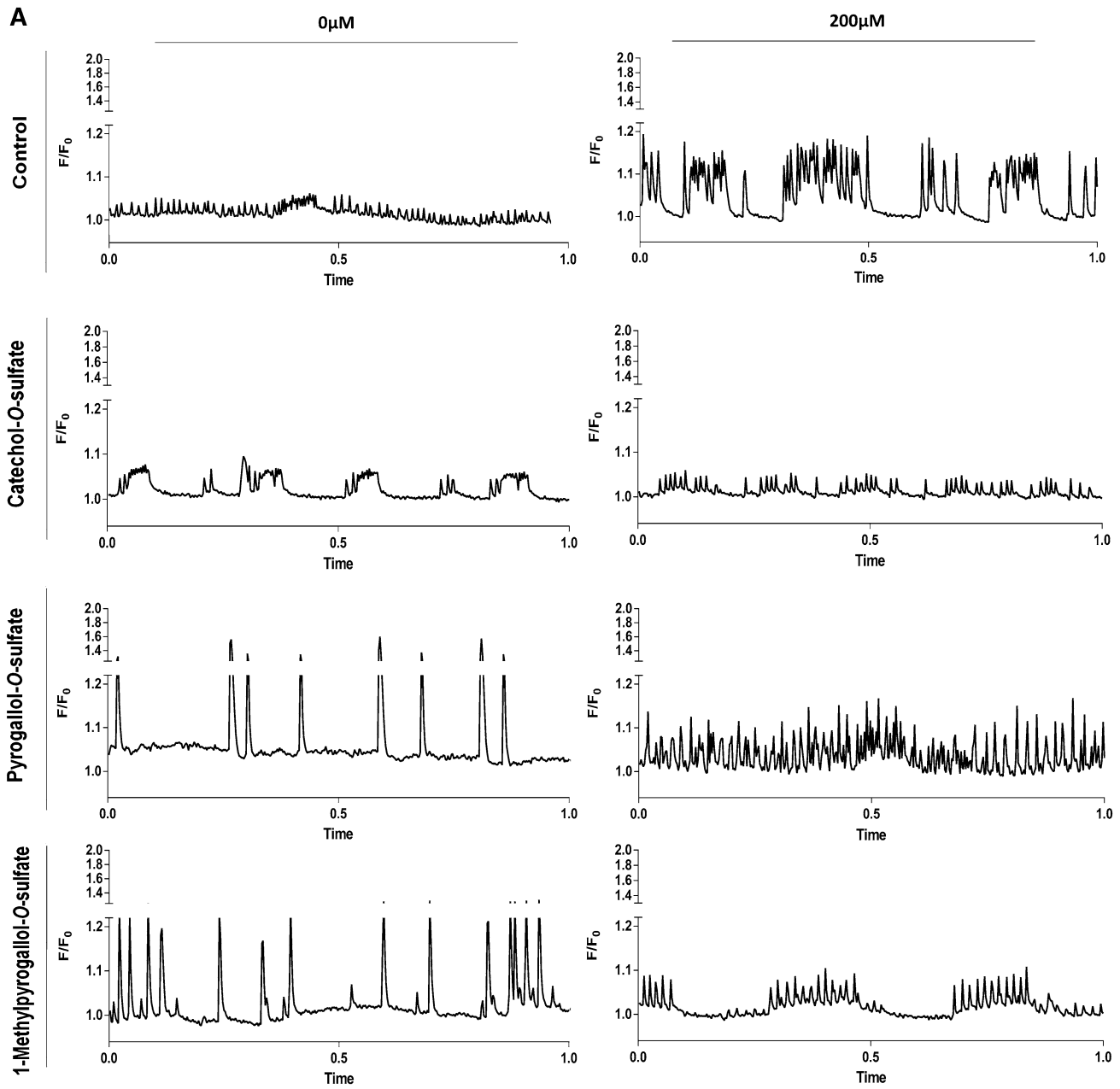

Fig. 5 Ca^{2+} transients. **a** Representative profile of cytosolic calcium transients in neonatal rat cardiomyocytes, after a 2-h pre-treatment with phenolic metabolites and exposure for 24 h with 200 μM ISO. **b** Calcium transients' amplitude (F/F_0) in neonatal rat cardiomyocytes. F stands for fluorescence measure at time t and F_0 stands for mean baseline fluorescence, calculated from several frames collected between beats. Filled circle represents a calcium transient and the red bar stands for the mean calcium transient's amplitude. Data from at least three independent experiments. In each condition, transient amplitudes of ten cells were measured per independent experiment. $^{****}P<0.0001$ versus Control. $^{\#\#\#\#}P<0.0001$ versus ISO (*ISO* isoproterenol)

(Fig. 5a, b). Whenever cardiomyocytes were pre-treated with the phenolic metabolites and challenged with ISO, there is a decrease in the harmful effect of the β-AR agonist. Indeed, these phenolic metabolites promoted a more uniform profile of Ca^{2+} transients (Fig. 5a). Likewise, they significantly reduced the amplitude of Ca^{2+} transients up to values similar to non-treated cardiomyocytes (Fig. 5b).

Cardiomyocytes exposed only to catechol-*O*-sulphate also generate longer Ca^{2+} transients, with longer pauses between cardiomyocyte beats (Fig. 5a), but without any significant alteration on amplitude of Ca^{2+} transients (Fig. 5b). Likewise, pyrogallol-*O*-sulphate and 1-methylpyrogallol-*O*-sulphate-exposed cardiomyocytes presented a decrease in the number of Ca^{2+} transients throughout the protocol (Fig. 5a),

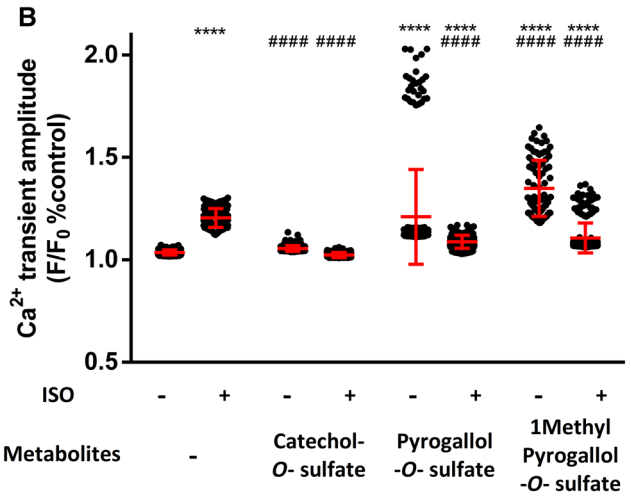

Fig. 5 (continued)

with a significant increase of Ca^{2+} transients' amplitude (Fig. 5b). It indicates that these phenolic metabolites might be able to directly modify Ca^{2+} signalling in cardiomyocytes. Moreover, there was some heterogeneity in the Ca^{2+} amplitudes of cells exposed to pyrogallol-*O*-sulphate and 1-methylpyrogallol-*O*-sulphate. These data seem to be in accordance with the direct activation of CaMKII (Fig. 4b) by pyrogallol-*O*-sulphate and 1-methylpyrogallol-*O*-sulphate, which may also contribute to the recorded unsynchronized beating (Fig. 2b). Thus, the Ca^{2+} transients' results are in accordance with the modulation of cell beating by the phenolic metabolites.

In summary, generated data indicate these three human bioavailable phenolic metabolites are able to modulate Ca^{2+} transients' occurrence and amplitude in response to adrenergic stress, consequently promoting a more synchronous cardiomyocytes' beating profile similar to control cardiomyocytes.

Discussion

Due to the increasing incidence of CVD, polyphenols have been in the spotlight as potential efficient and cost-effective approaches to limit and eventually to treat CVD, with an important social and economic impact [14–18]. Pimpão et al. have recently identified and quantified human bioavailable phenolic metabolites present in human-circulating plasma, following consumption of a specific berries' purée [21, 22]. Herein, the great novelty is the use some of the human bioavailable phenolic metabolites identified by Pimpão et al. in cardiomyocyte cultures for the assessment of polyphenols' cardioprotective properties. Neonatal rat cardiomyocytes and differentiated cardiomyoblasts were exposed to the three most abundant phenolic metabolites present in human plasma: catechol-*O*-sulphate, pyrogallol-*O*-sulphate and 1-methylpyrogallol-*O*-sulphate [22]. These human bioavailable phenolic metabolites were tested under the concentration and time they were found circulating in the human plasma. Thus, this strategy allows a physiologically more relevant approach to search for cardioprotective properties of polyphenols. Although primary cultures of adult cardiomyocytes are a more representative in vitro model, these cells are more prone to cell death when cultured in vitro, which limit their interest for mechanistic studies of cytoprotective molecules. Thus, the used model herein was neonatal rat cardiomyocyte cultures.

Activation of β-AR increases contractility and relaxation of cardiomyocytes and heart rate via phosphorylation and activation of PKA, followed by phosphorylation of other key proteins involved in Ca^{2+} dynamics and excitation–contraction coupling [2, 3]. Although the sympathetic β-AR system is crucial to respond against acute stressful situations, chronic stimulation of these receptors is harmful, resulting in arrhythmogenic cell beating behaviour of cardiomyocytes [1–4]. Likewise, it has been described that chronic exposition to ISO generates arrhythmogenic Ca^{2+} transients and increased ROS levels in cardiomyocytes [8, 9]. Herein, ISO treatment of cardiomyocytes for 24 h was used as an in vitro model of cardiac stress mimicking continued activation of β-AR. The potential cardioprotective role of polyphenols was evaluated by treating cardiomyocytes with phenolic metabolites for 2 h prior to ISO exposure.

Our data showed that these three phenolic metabolites reduced mitochondrial oxidative stress promoted by prolonged β-AR stimulation, which is similar to other polyphenols previously described to be cardioprotective and antioxidant [14].

ISO-induced β-AR stimulation for 24 h decreased cardiomyocyte beating and synchrony. These effects were reverted whenever cardiomyocytes were pre-treated with the phenolic metabolites. For the first time, modulation of cardiomyocyte beating was described as an event implicated in the beneficial cardioprotective role of polyphenol-derived metabolites.

A tight control of Ca^{2+} signalling is crucial for the well-functioning of cardiomyocytes. Activation of β-AR and consequent activation of the PKA/CaMKII pathway promotes Ca^{2+} transients, stimulating cellular contraction [2, 3, 5, 6]. Our results showed that phenolic metabolites limited PKA/CaMKII phosphorylation and activation under adrenergic stressful conditions. It has been widely described that the activation of PKA/cAMP pathway and CaMKII promotes the increased phosphorylation of ryanodine receptors [2, 3, 5, 6]. This leads to an increase of Ca^{2+} release from the sarcoplasmic reticulum. Furthermore, activation of PKA and CaMKII also decreases the inhibitory effect of phospholamban on the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA),

which enhances the uptake of Ca^{2+} by SERCA into the sarcoplasmic reticulum [2, 3, 5, 6]. The consequent higher rates of Ca^{2+} release and uptake enhance cardiomyocytes' cell beating frequency [2–5]. Nevertheless, prolonged adrenergic stimulation and subsequent overactivation of PKA and CaMKII lead to decompensated Ca^{2+} handling, sarcoplasmic reticulum Ca^{2+} leakage, and cardiac arrhythmic features [7–9]. Moreover, results from Bovo et al. indicate that oxidation of the ryanodine receptors, alongside the receptors' phosphorylation, will enhance the RyR activity, increasing the occurrence of arrhythmogenic calcium waves [8, 9]. Thus, by attenuating PKA/CaMKII activation and by limiting mitochondrial ROS generation, these phenolic metabolites might decrease the phosphorylation and/or activity of downstream proteins of PKA and CaMKII pathways, particularly RyR, in cells exposed to ISO. Further research is needed to confirm the direct involvement of RyR on polyphenol-induced cardioprotection.

Normal beating rate and contractility/relaxation of cardiomyocytes are directly dependent on Ca^{2+} transients' amplitude and frequency [2, 3, 5, 6]. Thus, Ca^{2+} transients were assessed in the presence of the three phenolic metabolites with ISO treatment. Prolonged stimulation of β-AR increased Ca^{2+} transients' amplitude and promoted their unsynchronized frequency. Pre-treatment with three metabolites reverted the ISO-induced increase on Ca^{2+} transients' amplitude. Nevertheless, 1-methylpyrogallol-*O*-sulphate decreased Ca^{2+} transients' amplitude but under an unsynchronized manner. Taken all together, the tested phenolic metabolites improved Ca^{2+} transients in response to adrenergic stress by modulating amplitude and/or synchronization, which might then result in the modulation of cardiomyocytes' beating.

Interestingly, human bioavailable phenolic metabolites also modulate cardiomyocyte beating and Ca^{2+} signalling under normal conditions, i.e. in the absence of ISO treatment and adrenergic stress. In fact, the three tested metabolites promoted a larger distribution in beating cell population, indicating a more asynchronous beating pattern. Likewise, 1-methylpyrogallol-*O*-sulphate decreased cardiomyocyte beating to levels similar to ISO-treated cells. Accordingly, CaMKII phosphorylation and activation also occurred in response to the three metabolites. These results suggest that phenolic metabolites might also directly activate CaMKII kinase in a PKA-independent way. Furthermore, and in accordance with beating results, 1-methylpyrogallol-*O*-sulphate activates PKA at similar levels of ISO treatment. Finally, cardiomyocytes treated with pyrogallol-*O*-sulphate or 1-methylpyrogallol-*O*-sulphate presented higher Ca^{2+} transient amplitude and heterogeneity.

Thus, taken all together, these data indicate phenolic metabolites directly modulate cardiomyocyte beating and synchrony, and Ca^{2+} signalling. And whenever applied along with adrenergic stressful stimulation, these metabolites become cardioprotective by reducing ROS generation, modulating beating rate, controlling of PKA/CaMKII pathway and improving regulation of Ca^{2+} transients' amplitude and frequency.

Catecholamines bind to β-AR with a higher or lower degree of efficacy, depending on the compound's chemical nature [34, 35]. Thus, catechol and pyrogallol have already been described as agonist of elements of the G protein-coupled receptors (GPCR) family, including β_2-adrenergic receptors [36, 37]. One may speculate that the three phenolic metabolites may directly interact with the β-AR.

Despite β_1-adrenergic receptors being the predominant receptors in cardiomyocytes, catecholamines may also interact with other subtypes of receptors that do not follow the classic PKA/cAMP signalling pathway [37]. In a context of exacerbated catecholamine levels, it has been described a downregulation of β_1-adrenergic receptors and an upregulation of β_3-adrenergic receptors in failing human hearts [38]. And these two receptors have a cross-modulation mechanism between them [39].

Thus, alterations in cardiomyocytes' beating and Ca^{2+} signalling after catechol-*O*-sulphate, pyrogallol-*O*-sulphate and 1-methylpyrogallol-*O*-sulphate exposure may also be due to the balance between their interactions with different subtypes of β-AR. Another hypothesis is that metabolites could desensitize β-AR, by interacting with them [40, 41]. Nevertheless, if these phenolic metabolites desensitized β-AR, they would not revert the ISO effect by increasing cell beating and synchronization.

Moreover, modulation of cardiomyocyte membrane's ionic currents can alter Ca^{2+} signalling and the contraction-relaxation behaviour of cells. One may also speculate that these three metabolites may interact with ionic channels present in the membrane of cardiomyocytes, as other polyphenols have been described to do [42–46]. By modulating ionic channels currents, these compounds may alter Ca^{2+} currents and/or Ca^{2+} signalling, altering the cell beating of neonatal rat cardiomyocytes [42–46]. Further research is needed to fully clarify possible modulation of cardiomyocytes' ionic currents by the phenolic metabolites.

This work aims at exploring the molecular mechanisms underlying the putative cardioprotective effects of berry-derived polyphenol metabolites. This knowledge is important for future nutritional or therapeutic approaches using polyphenols against CVD. However, there is still the need to further clarify the efficacy of these compounds in human clinical trials, as well as their safety when taken in combination with other drugs [47].

For the first time, the potential cardioprotective role and their underlying molecular mechanisms of polyphenols were assessed by using their corresponding human bioavailable phenolic metabolites, under physiological conditions.

Phenolic metabolites promote cardioprotection by improving cardiomyocytes' beating and Ca^{2+} signalling in response to continued stimulation to β-AR.

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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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