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Protective role of the novel hybrid 3,5-dipalmitoyl-nifedipine in a cardiomyoblast culture subjected to simulated ischemia/reperfusion

Eduarda Santa-Helena[a,b], Stefanie Teixeira[b], Micheli Rosa de Castro[a], Diego da Costa Cabrera[c], Caroline Da Ros Montes D'Oca[c], Marcelo G. Montes D'Oca[c], Ana Paula S. Votto[a,b], Luiz Eduardo Maia Nery[a,b,*,1], Carla Amorim Neves Gonçalves[a,b]

[a] Graduate Program in Physiological Sciences, Comparative Animal Physiology, Institute of Biological Sciences, Universidade Federal do Rio Grande, FURG, Rio Grande, RS, Brazil
[b] Institute of Biological Sciences, Universidade Federal do Rio Grande, FURG, Rio Grande, RS, Brazil
[c] Kolbe Organic Synthesis Laboratory, School of Chemistry and Food, FURG, Rio Grande, RS, Brazil

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ABSTRACT

This work investigated the acute effects of the calcium channel blocker nifedipine and its new fatty hybrid derived from palmitic acid, 3,5-dipalmitoyl-nifedipine, compared to endocannabinoid anandamide during the process of inducing ischemia and reperfusion in cardiomyoblast H9c2 heart cells. The cardiomyoblasts were treated in 24 or 96-well plates (according to the test being performed) and maintaining the treatment until the end of hypoxia induction. The molecules were tested at concentrations of 10 and 100 μM, cells were treated 24 h after assembling the experimental plates and immediately before the I/R. Cell viability, apoptosis and necrosis, and generation of reactive oxygen species were evaluated. Nifedipine and 3,5-dipalmitoyl-nifedipine were used to assess radical scavenging potential and metal chelation. All tested molecules managed to reduce the levels of reactive oxygen species compared to the starvation + vehicle group. In in vitro assays, 3,5-dipalmitoyl-nifedipine showed more antioxidant activity than nifedipine. These results indicate the ability of this molecule to act as a powerful ROS scavenger. Cell viability was highest when cells were induced to I/R by both concentrations of anandamide and the higher concentration of DPN. These treatments also reduced cell death. Therefore, it was demonstrated that the process of hybridization of nifedipine with two palmitic acid chains assigns a greater cardioprotective effect to this molecule, thereby reducing the damage caused by hypoxia and reoxygenation in cardiomyoblast cultures.

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1. Introduction

Cardiovascular disease is a global public health problem. Damage after myocardial ischemia and reperfusion (I/R) is the leading cause of morbidity and mortality globally [1,2]. Hypertensive patients are most affected by I/R [3], stressing the importance of studies investigating drugs that attenuate blood pressure while concurrently preventing or mitigating I/R myocardial damage.

Evidence indicates that several interrelated factors, such as the decrease in cellular ATP levels, the production of reactive oxygen species (ROS), the accumulation of hydrogen ions and the generation of reactive nitrogen species, contribute to the damage caused by I/R [4]. Oxidative stress contributes to the cascade of events leading to cell death: increased ROS production may modify the expression of various inflammatory mediators during cardiac injury [5]. During reoxygenation, the membrane integrity is compromised by the oxidation of the phospholipids which leads to uncontrolled ion permeability [6]. The ROS can still compromise the function of cardiac proteins, such as ion channels, calcium pumps and contractile proteins involved in the excitation mechanism of heart contraction [7]. Thus, the restoration of blood flow and the return of the oxygen supply generate ROS that trigger cell death through apoptosis and necrosis [6].

Substances that have anti-hypertensive and antioxidant action can bring benefits for the treatment of hypertensive patients prone to ischemia and reperfusion injury. According to Benzie and Tomlinson [8], antihypertensive drugs such as captopril, fosinopril,

* Corresponding author at: Institute of Biological Sciences, Universidade Federal do Rio Grande, FURG, Av. Itália Km 8, CEP 96201-900, Rio Grande, RS, Brazil.
E-mail address: famnery@terra.com.br (L.E.M. Nery).
[1] Institute of Biological Sciences, Universidade Federal do Rio Grande, FURG, Rio Grande, RS, 96203-900, Brazil.

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enalapril, perindopril, quinapril, and ramipril can act as iron ion scavengers, using the ferric reduction antioxidant power test (FRAP), although these substances are not as powerful as the classic antioxidant ascorbic acid. An antioxidant substance can protect biomolecules from damage mediated by both in vivo and in vitro free radicals by preventing or slowing oxidation of macromolecules. It does this by acting against the toxicity of metals, connecting these metals and avoiding reactive oxygen species generation. It also works through the chelation of those metals maintaining the redox state of the molecule [8].

Another commonly used antihypertensive drug is nifedipine (NIF) [9]. It is a member of the dihydropyridine family, which is characterized by blocking the calcium channel, and is also a potent vasodilator that is widely used as an antihypertensive [9]. However, one of the side effects of nifedipine is tachycardia, a risk factor for ischemia diseases such as acute myocardial infarction [10,11]. The treatment of cardiovascular diseases with hybrid molecules may involve more than one pharmacological action in a single drug, thereby providing an efficient alternative to potentiate already known effects or assign new effects to molecules [10].

NIF is also effective in inhibiting the activation of tumor necrosis factor kappa beta (NFkB), thereby contributing to decreased inflammation, and increased endothelial function in coronary circulation [12,13]. NIF has been shown to promote re-endothelialization after vascular injury, and is considered to protect against atherosclerosis by inhibiting endothelial cell apoptosis and suppressing vascular inflammation, effects attributed to the antioxidant properties of the drug [13,14].

Previous studies have shown the relationship between cannabinoid receptors and oxidative stress, confirming the antioxidant action of the ligands of these receptors in human pancreatic tumor cell lines [15–17]. However, the antioxidant effect of endocannabinoids is not only connected to their receptors. Some studies have shown that endocannabinoids play an important modulatory role in the function of the cardiovascular system in various pathological conditions, such as hypertension, myocardial infarction and heart failure [16,17].

To assist in mitigating the damage caused by I/R, we report in this study a convenient synthesis of novel hybrid molecule, 3,5-dipalmitoyl-nifedipine (DPN), via a one-pot Hantzsch multicomponent reaction using sulfamic acid as an inexpensive and nontoxic catalyst (Fig. 1). In the process of joining molecules, the fatty amides show an essential biological activity, whose importance is likely to facilitate the permeability of the cells of these new molecules [18]. Since the myocardial ischemia process is a global health problem, the authors aimed to study the acute effects of the calcium channel blocker nifedipine and its hybrid fatty acid during hypoxia/reoxygenation in a H9c2 cardiac cell line (cardiomyoblast) and compare these effects with the effect of anandamide, as well as in vitro testing the antioxidant potential of nifedipine and its hybrid fatty acid.

2. Methodology

2.1. Apparatus and chemistry

Sulfamic acid (98%) was supplied by Aldrich Chemical Co., and the methanol was supplied by Merck. The other reagents were purchased from Aldrich Chemical Co. and used without further purification. All organic solvents used for the synthesis were of analytical grade. The palmitic β-keto esters were synthesized through transesterification of methyl acetoacetate with the respective alcohol derived from palmitic (C16:0) acid. Anandamide (AEA) was synthesized by reacting arachidonic acid with ethanol amine, triethylamine, and a catalytic amount of dimethylaminopyridine (DMAP), and dicyclohexylcarbodiimide (DCC). Nifedipine was synthesized using the same experimental protocol described for 3,5-dipalmitoyl-nifedipine (DPN) in the item 2.2. The spectroscopy data used for anandamide (AEA) and nifedipine (NIF) are according to the literature. The reactions were monitored using thin-layer chromatography (TLC) performed with plates containing silica gel (Merck 60GF245), and the spots were visualized using iodine. Column chromatography was performed using Silica Gel 60 A (ACROS Organics, 0.035–0.070 mesh). Yields refer to chromatographically and spectroscopically homogeneous materials. The NMR spectra were recorded using a Varian VNMRS 300 spectrometer (^{1}H at 300 MHz and ^{13}C at 75.5 MHz) and deuterochloroform ($CDCl_3$) as the solvent. The chemical shift data are reported in units of δ (ppm) downfield from tetramethylsilane (TMS), which was used as an internal standard.

2.1.1. Experimental procedure for synthesizing 3,5-dipalmitoyl-nifedipine (DPN)

Palmitic β-keto ester (2 mmol), 2-nitro-benzaldehyde (1 mmol), ammonium acetate (3 mmol), methanol (5 mL) and

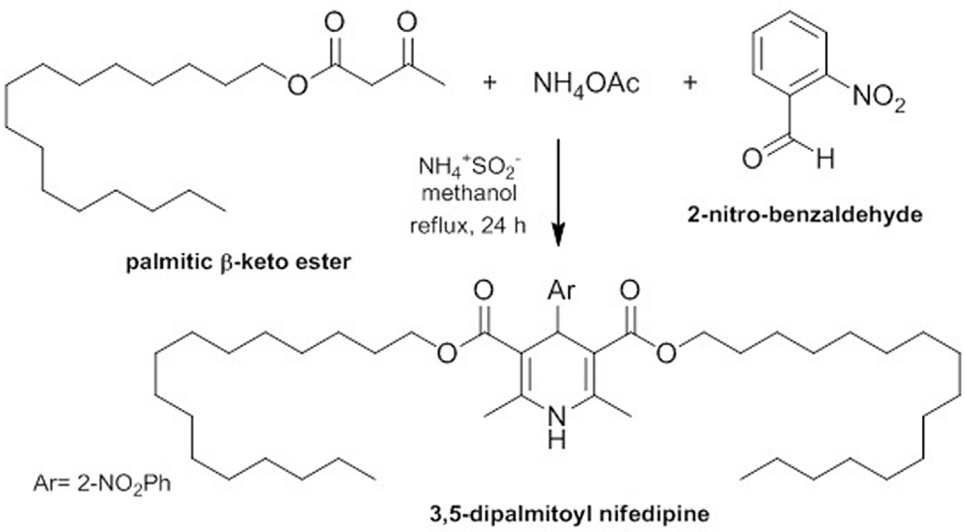

Fig. 1. Synthesis of the new hybrid 3,5-dipalmitoyl-nifedipine (DPN) through a Hantzsch multicomponent reaction using sulfamic acid.

sulfamic acid (30 mol%, as a catalyst) were added to a round-bottom flask equipped with a magnetic stir bar and reflux condenser. The mixture was subjected to reflux for 24 h. The crude product was then cooled to ambient temperature, concentrated in vacuum and purified through column chromatography using hexane/ethyl acetate (80:20 ratio) as an eluent, resulting in a pure yellow paste compound.

Dihexadecyl 2,6-dimethyl-4-(2-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate (DPN):

MW 767 g mol^{-1}; Yellow paste; Yield: 75%. NMR ^{1}H (300 MHz, $CDCl_3$) d 0.88 (t, 6H, J = 6.0 Hz), 1.26 (m, 52H), 1.54 (m, 4H), 2.31 (s, 6H), 3.94 (m, 2H), 4.06 (m, 2H), 5.82 (s, 1H), 5.97 (bs, 1H), 7.23 (m, 1H), 7.45 (t, 1H, J = 7.5 Hz), 7.53 (d, 1H, J = 9.0 Hz), 7.72 (d, 1H, J = 9.0 Hz); ^{13}C (75 Mz, $CDCl_3$) d 14.5 (2C), 19.9 (2C), 23.0 (2C), 26.2 (3C), 28.9 (4C), 29.6 (3C), 29.7 (3C), 29.9 (4C), 30.0 (6C), 32.2 (3C), 35.0, 64.6 (2C), 104.1 (2C), 124.3, 127.2, 131.6, 133.0, 142.9, 144.9 (2C), 148.2, 163.3 (2C); IR(film, $\nu_{max}cm^{-1}$): 758, 1215, 1306, 1354, 1489, 1531, 1689, 2854, 2926, 3018, 3346, 3437.

2.1.2. Cell cultures

Wistar rat H9c2 cardiomyoblast embryo cells were obtained from the Rio de Janeiro Cell Bank (Rio de Janeiro, Brazil) and maintained in culture bottles at 37 °C in Dulbecco's Modified Eagles Medium (DMEM). This was supplemented with sodium bicarbonate (1.5 g/L), L-glutamine (4 mM), Hepes (25 mM), glucose (4.5 g/L), 1% antibiotic (penicillin 100 U/ml and streptomycin 100 μg/ml) and an antimycotic agent (amphotericin 0.25 μg/ml), and 10% fetal bovine serum was added. Cells were maintained in a manner that did not exceed 80% confluence.

2.1.3. Experimental design

The H9c2 cells (1 × 10 cells/mL) were placed in 24- or 96-well plates (according to the test performed), with a DMEM culture medium at 37 °C for 24 h for adhesion. At the end of this period, the cells were distributed in the following groups: control (in DMEM culture medium), I/R (in culture medium DMEM + induction of hypoxia and reoxygenation), starvation (in PBS), starvation + I/R (in PBS + induction hypoxia and reoxygenation), starvation + vehicle (in PBS + 1% dimethyl sulfoxide, DMSO), anandamide (AEA + starvation), Anandamide + I/R (AEA + starvation + vehicle + induction of hypoxia and reoxygenation), nifedipine (NIF + starvation), nifedipine + I/R (NIF + starvation + induction of hypoxia and reoxygenation), 3,5-dipalmitoyl-nifedipine (DPN + starvation) and 3,5-dipalmitoyl-nifedipine + I/R (DPN + starvation + induction of hypoxia and reoxygenation). All groups were tested with the induction of hypoxia and reoxygenation (groups I/R) or in the absence of this condition. The molecules were administered at concentrations of 10 and 100 μM, always 24 h after assembling the experimental plates and immediately before the I/R induction.

2.1.4. Induction of ischemia and reoxygenation (I/R)

The ischemia conditions were obtained by replacing the culture medium with the experimental medium, which consisted of a PBS buffer (pH 6.8 at 37 °C), and the plates were incubated in a hypoxic chamber with nitrogen passage coupled to a vacuum pump to decrease the oxygen pressure. After 30 min of hypoxia, PBS was replaced with DMEM, and normoxic conditions were restored by subjecting the plates to the reoxygenation period for another 30 min.

2.1.5. Generation of reactive oxygen species (ROS)

After reoxygenation, the cardiomyoblast and controls were suspended using trypsin and then placed in eppendorf tubes and incubated at 37 °C for 30 min in PBS with diacetate 2′,7′-diclorofluorescein (H_2DCF-DA) in a final concentration of 40 μM. After being incubated with H_2DCF-DA, the cells were washed with PBS once and resuspended. For each sample, aliquots of 160 μL were placed in triplicate in a 96-well plate, and the fluorescence intensity was determined for 90 min at 37 °C in a Victor 2 fluorometer (Perkin-Elmer), with excitation wavelengths of 485 nm and emission at 520 nm. The ROS levels were expressed in fluorescence units.

2.1.6. In vitro analyses

2.1.6.1. DPPH photometric assay. The effect of DPN and NIF on DPPH (2,2-diphenyl-1-picrylhydrazyl) radicals was measured using the modified method of Sharma and Bhat [19]. The compounds were diluted to final concentrations of 1, 10, 100 and 200 μM. The reaction mixture was shaken thoroughly and incubated for 30 min at 30 °C in the dark, and the absorbance was measured at 517 nm against a blank. The IC_{50} value (μM) is the effective concentration at which DPPH radicals were scavenged by 50%. The radical scavenging activity was calculated using the following equation:

$$\text{Scavenging effect (\%)} = (A_{control} - A_{sample}/\ A_{control}) \times 100$$

Where $A_{control}$ is the absorbance of the control (without compound).

2.1.6.2. ABTS radical scavenging. The determination of the radical scavenging effect of DPN and NIF on ABTS (2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) – $ABTS^{\bullet}$+) radicals was performed according to the method of [20], with some modifications. Briefly, ABTS radical was added to a medium containing DPN and NIF (1, 10, 100 e 200 μM). The media were incubated for 30 min at 25 °C. The decrease in absorbance was measured at 734 nm, depicting the scavenging activity of compounds against the ABTS radical. Results are expressed as percentage of the blank (without compound). The radical scavenging activity was calculated following the same equation used in the DPPH assay (see Section 2.1.6.1).

2.1.6.3. Ferric ion reducing antioxidant power (FRAP) assay. The FRAP assay was carried out as described by Benzie et al. [21], with slight modifications. The FRAP reagent was prepared by mixing 38 mM sodium acetate (anhydrous) in milli Q water pH 3.6, 20 mM $FeCl_3 \cdot 6H_2O$ in milli Q water and 10 mM 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) in 40 mM HCl in proportions of 10:1:1. This reagent was freshly prepared before each experiment. Different concentrations of DPN and NIF and FRAP reagent were added to each sample, and the mixture was incubated at 37 °C for 40 min in the dark. The absorbance of the resulting solution was measured at 593 nm by a spectrophotometer. FRAP values were expressed as absorbance.

2.1.7. Cell viability

Cell viability was evaluated using the MTT method (3-[4,5-dimethylthiazol-2-yl]-2,5-difeniltetrazolium). Briefly, cells were rinsed with PBS and added to a DMEM, with 10% MTT, and then incubated for 3 h at 37 °C. The DMEM was then removed, and formazan crystals were dissolved in 200 μL of DMSO (Sigma), and its absorbance values were immediately read at 490 nm in a spectrophotometer with a plate reader (800 ELX Universal Microplate Reader, Bio-TEK). The values were expressed as arbitrary values.

2.1.8. Analysis of apoptosis and necrosis

Apoptosis and necrosis were evaluated according to Ribble [22] (modified). To analyze cell apoptosis and necrosis, after reoxygenation, 2 μL of a working solution composed of PBS, 100 μg/ml

acridine orange and 100 μg/ml ethidium bromide was added. An epifluorescence microscope was used to analyze the captured images from each well of the plate (20×) with (Olympus IX81). Cells were analyzed using the Image J program, and the data were expressed as the number of cells counted and the percentage of viable cells in apoptosis or necrosis in relation to the total number of cells in each well. Cells were classified according to Kosmider [23].

2.1.9. Statistical analysis

Each experiment was repeated at least three independent times for each sample. The results were expressed as the mean ± SE. The assumptions of normality (Kolmogorov-Smirnov test) and variances of homoscedasticity (Levene test) were tested. One-way analysis of variance (ANOVA) was used to determine significant between-group differences, followed by Tukey's HSD post hoc test. The level of statistical significance was set at 5%.

3. Results

3.1. Induction of ischemia and reoxygenation

To confirm the effectiveness of ischemia and reperfusion, a simulation protocol initially tested the effect of I/R and starvation, separately and together. Our results showed that when the cell line H9c2 is exposed only to I/R, there is an increase of 50.7% in the generation of reactive oxygen species in the control group (Fig. 2A) and a significant decrease in cell viability by 45% compared to the control group (Fig. 2B).

These differences are even greater when the cells are subjected to I/R and starvation. In this condition, there is a 68.1% and 75% increase compared to the control, in ROS production (Fig. 2A) and cell viability (Fig. 2B), respectively. In the analysis of apoptosis and necrosis, we also observed that when the cells were subjected to I/R or starvation, death by necrosis (Fig. 2C) was greater when the treatments were combined (i.e., I/R + starvation). Apoptosis was

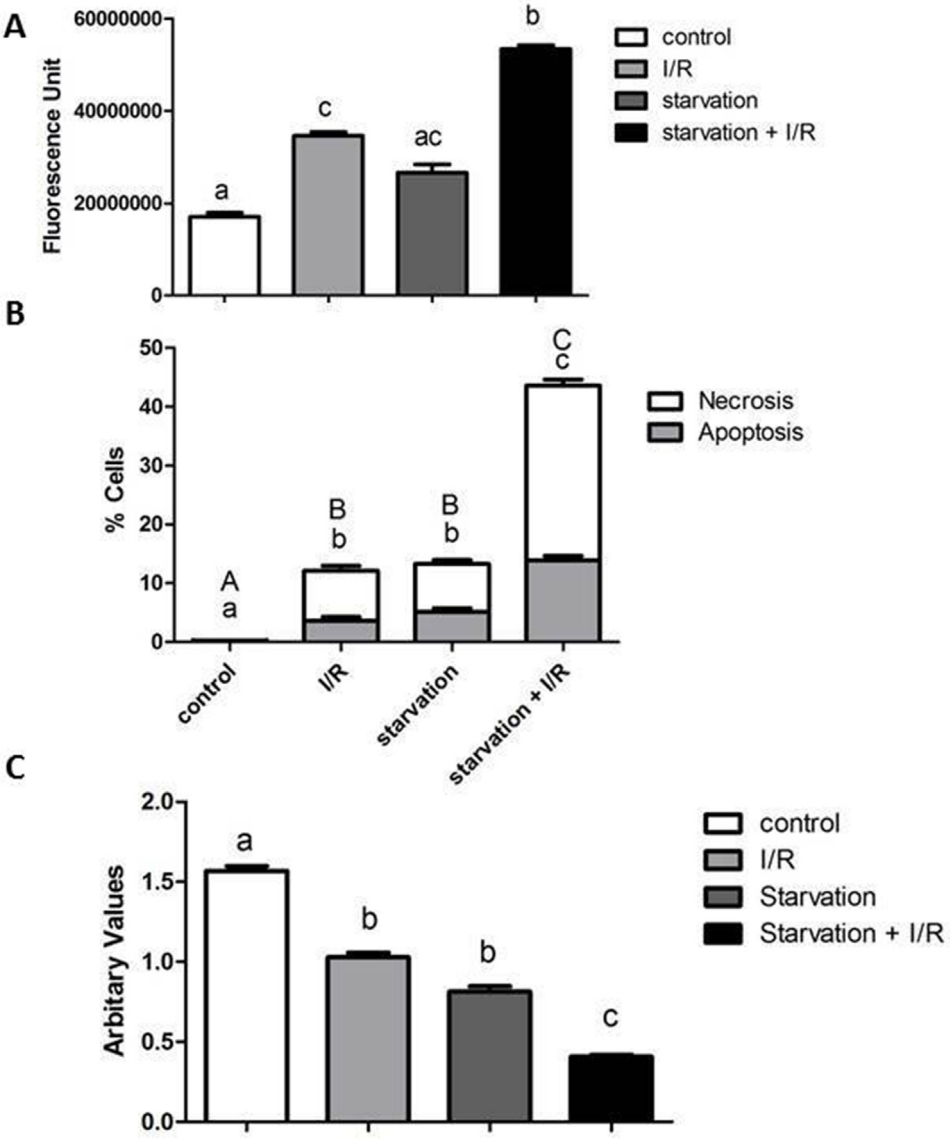

Fig. 2. Groups are: control (in DMEM culture medium), I/R (in culture medium DMEM + induction of ischemia and reoxygenation), starvation (in PBS), starvation + I/R (in PBS + induction ischemia and reoxygenation). (A) ROS determination in H9C2 cells subjected to the ischemia and reoxygenation protocol. (B) Percentage of cell viability immediately after treatment with the ischemia and reoxygenation protocol (C) Percentage of viable, apoptotic and necrotic H9c2 cells immediately after treatment. Data are expressed as the mean ± standard error. Different letters indicate significant difference between groups ($p < 0.05$). Uppercase letters in C refer to differences between groups of necrotic cells, and lowercase letters refer to differences between groups of apoptotic cells ($p < 0.05$).

induced significantly ($p < 0.05$) with the I/R + starvation treatment. These results demonstrate that our protocol of I/R + starvation was effective at increasing the amount of reactive oxygen species and decreasing cell viability by simulating the damage induced by ischemia and reoxygenation.

3.1.1. Cardioprotective effect of the treatments

The results showed that when subjected to starvation only (Fig. 3) in both AEA concentrations (10 μM and 100 μM) and the highest concentration of 3,5-dipalmitoyl-nifedipine (DPN) (100 μM), the H9c2 cells showed reduced ROS levels (33.75%, 40.05% and 29.20%, respectively) compared with the starvation + vehicle, however, when subjected to I/R + starvation, all molecules show a reduction in reactive oxygen species ($p < 0.05$). This decrease was more intense in both concentrations of AEA (10 μM and 100 μM) 30% and 36.30%, respectively, and DPN 100 μM (27.9%) (Fig. 3B).

As shown in Fig. 4, the DPN compound exhibited scavenger activity at concentrations equal to or greater than 1 μM in the ABTS radical scavenging assays at different concentrations with IC_{50} values of 2.45 (1.69–3.55) μM and maximum inhibition (% Imax) of 81.24. The NIF compound exhibited scavenger activity from 10 μM up to greater concentration with IC_{50} values of 28.78 (21.91–37.81) μM and maximum inhibition (% Imax) of 86.18.

The ferric reduction power of a compound may serve as a significant indicator of its potential electron transfer ability, DPN and NIF demonstrated significant scavenger activity at 1 μM (Fig. 5). However, both molecules did not present DPPH radical scavenging activity at tested concentrations (data are not shown).

The viability of the H9c2 cell line, when subjected to starvation only (Fig. 6A), was significantly increased ($p < 0.05$), by 93.5% with AEA 10 μM, 110.06% with AEA 100 μM, 61.39% with DPN 100 μM and 44.25% with NIF 100 μM However, when these cells were induced through I/R + starvation (Fig. 6B), which is an ischemia

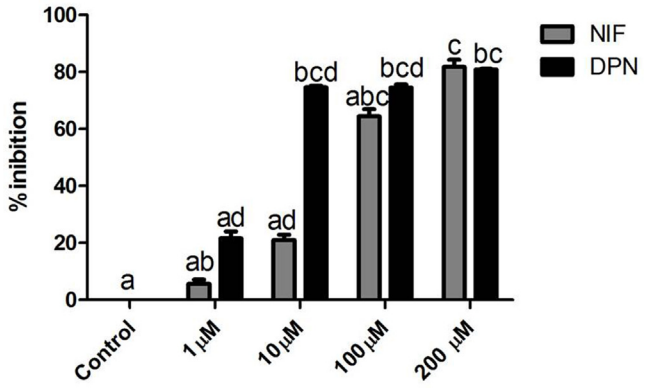

Fig. 4. ABTS assay with different concentrations of nifedipine (NIF) and novel hybrid molecule, 3,5-dipalmitoyl-nifedipine (DPN). The values are expressed in percentage of inhibition in relation to control (without compounds). The mean value of absorbance (734 nm) of the control is 0.66 ± 0.15. Each value is expressed as the mean ± SE ($n = 3$). Fluorescence unit data are expressed as mean ± standard error, and different letters indicate a significant between-group difference ($p < 0.05$).

condition, the cells treated with either anandamide or with 3,5-dipalmitoyl-nifedipine showed greater viability ($p < 0.05$) at both concentrations. This increase was 185.2% for 10 μM AEA, 209% for 100 μM AEA, 73.8% for 10 μM DPN, and 138.7% for 100 μM DPN. This increase in cell viability indicates a protective effect of these molecules from the damage caused by I/R. At concentrations of 10 μM and 100 μM, NIF did not differ significantly from starvation + vehicle during I/R ($p > 0.05$).

As shown in Figs. 5 and 7 A, H9c2 cells in the starvation group treated with nifedipine at both concentrations showed the highest values of apoptotic and necrotic cells, and these values are statistically equal to the starvation+ vehicle group ($p > 0.05$).

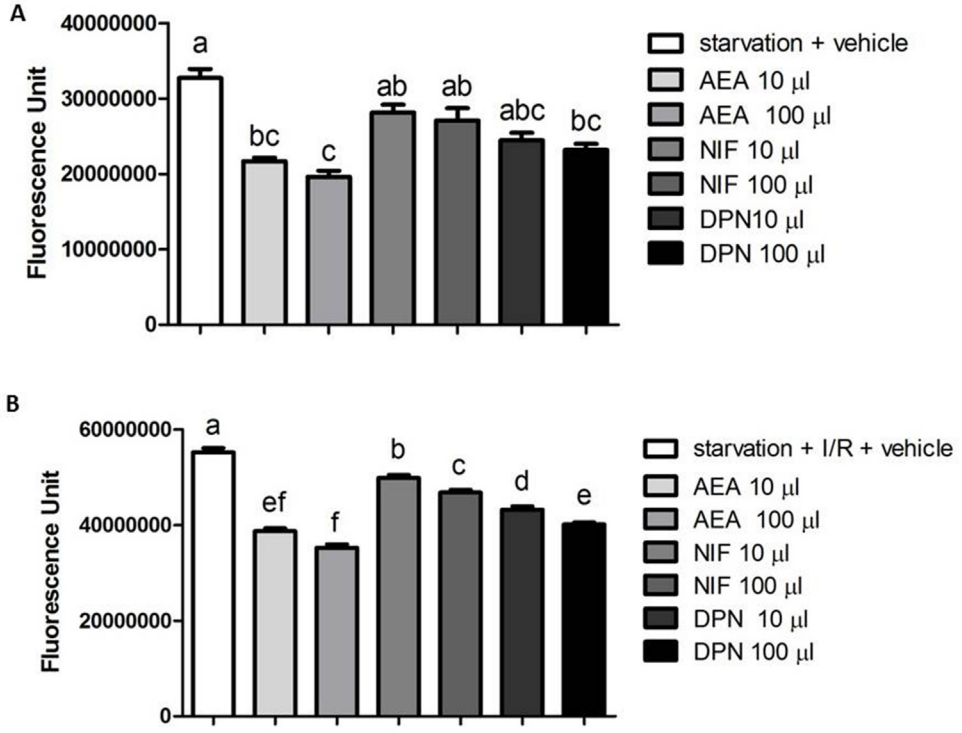

Fig. 3. Groups are: starvation + I/R (in PBS + induction hypoxia and reoxygenation), AEA (anadamide at different concentrations), NIF (nifedipine at different concentrations), DPN (3,5-dipalmitoyl-nifedipine at different concentrations). (A) ROS generation in H9C2 cells exposed to starvation, treated with anandamide, nifedipine and 3,5-dipalmitoyl-nifedipine, (B) ROS generation in H9C2 cells immediately before hypoxia, starvation and reoxygenation, treated with anandamide, nifedipine and 3,5-dipalmitoyl-nifedipine. Fluorescence unit data are expressed as the mean ± standard error, and different letters indicate a significant between-group difference ($p < 0.05$).

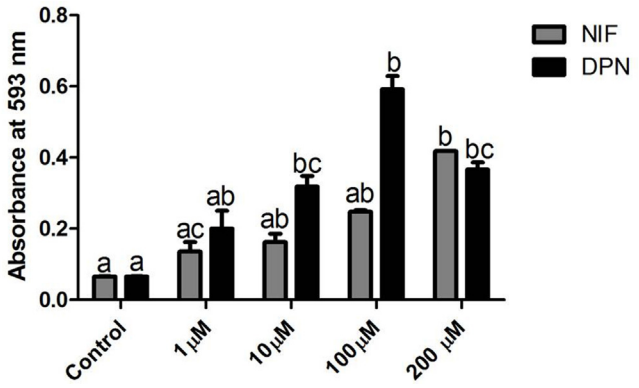

Fig. 5. Ferric ion reducing antioxidant power of DPN (3,5-dipalmitoyl-nifedipine) and NIF (nifedipine). Data are presented as the mean ± S.E of the absorbance values at 593 nm ($n = 3$). Fluorescence unit data are expressed as mean ± standard error, and different letters indicate a significant between-group difference ($p < 0.05$).

However, the AEA and DPN treatments in the two concentrations significantly decreased cell death ($p < 0.05$) either by necrosis or apoptosis. When H9c2 cells are subjected to I/R conditions (Fig. 7B), cells treated with both concentrations of anandamide and DPN 100 μM show a decreased incidence of death by apoptosis and necrosis. The two NIF concentrations and DPN 10 μM did not decrease death by apoptosis and necrosis.

4. Discussion

In the I/R condition, major damage to heart tissue occurs during reperfusion. Two factors may explain this: one attributes the damage to the large influx of Ca^{2+} into the cell, the other attributes the damage to oxidative stress [24]. In investigating cardioprotective drugs that reduce or prevent damage from ischemia and reperfusion [25,26], researchers have proposed new drugs with hybrid molecules that attenuate Ca^{2+} influx and oxidative stress [27–29].

The results showed that the presence of the two palmitic acids attached to the NIF, a classical calcium blocker channel, proving that the administration of DPN at concentrations of 10 and 100 μM in a rat cardiomyoblast cell line (H9c2) subjected to starvation and hypoxia/reoxygenation is capable of significantly reducing oxidative stress and increasing cell viability, in addition to decreasing cell death from apoptosis and necrosis.

Changes in mitochondrial function increase ROS and consequently, increase oxidative damage, possibly leading to a greater induction of apoptosis and necrosis, which are among the main causes of ischemia injury to the heart [30]. Evidence shows that an increase in the absorption of antioxidants in food and medicines can protect against cardiovascular disease [31].

The antioxidant effect attributed to NIF has already been well described in the literature [32,33]. NIF (5 μM) demonstrated an antioxidant effect in primary cultures of endothelial cells from human umbilical cord as well as in rat pheochromocytoma cells treated with 10 μM NIF [13,26]. Studies of homogenized heart rats treated with 2.7 mM NIF have also confirmed the antioxidant activity of this molecule [34]. In this work, it was observed that in cardiomyoblast cultures, NIF (10 and 100 μM) under I/R conditions also demonstrated antioxidant activity in a dose-dependent manner.

NIF is capable of improving functions related to angiogenesis in endothelial cells, such as differentiation and migration, in addition to improving the responses of cells to stress and decreasing cell death, for example [35]. According to Arora [36], NIF exhibits cytoprotective effects at a concentration of 10 μM. This result was also repeated when, at the same concentration, NIF protected

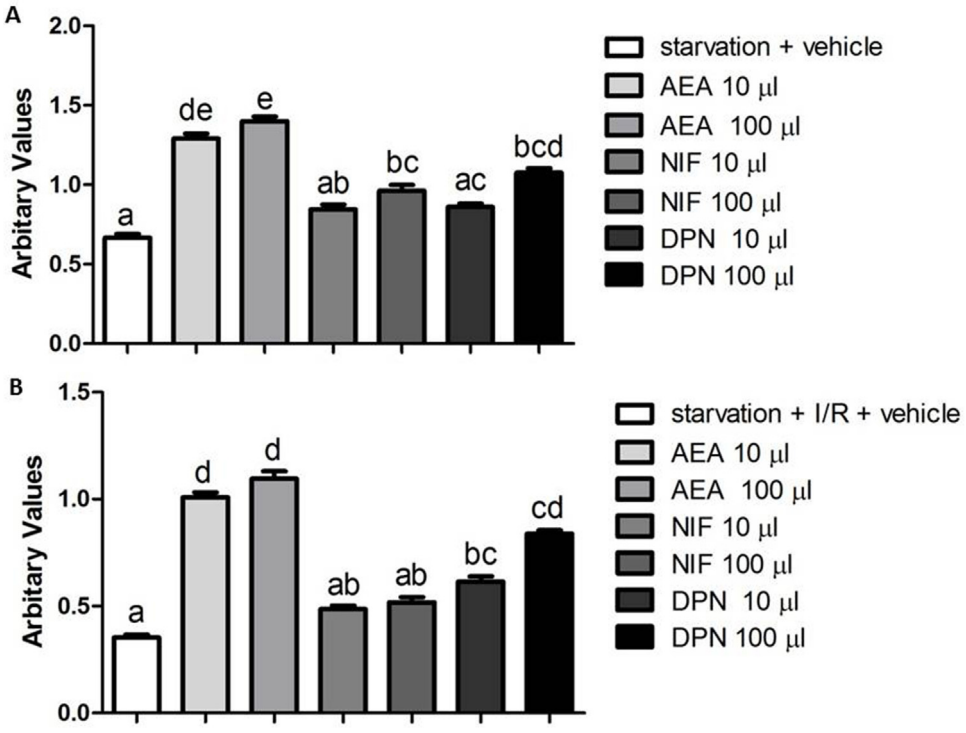

Fig. 6. Groups are: starvation + vehicle (in PBS + 1% dimethyl sulfoxide, DMSO) starvation + I/R + vehicle (in PBS + 1% dimethyl sulfoxide, DMSO + induction hypoxia and reoxygenation), AEA (anandamide at different concentrations), NIF (nifedipine at different concentrations), DPN (3,5-dipalmitoyl-nifedipine at different concentrations). (A) Cell viability of H9C2 cells exposed to starvation in the presence of anandamide, nifedipine and 3,5-dipalmitoyl-nifedipine, (B) Cell viability of H9C2 cells immediately treated with hypoxia and starvation in the presence of anandamide, nifedipine and 3,5-dipalmitoyl-nifedipine. All arbitrary data values are shown as the mean ± standard error, different letters indicate a significant difference ($p < 0.05$).

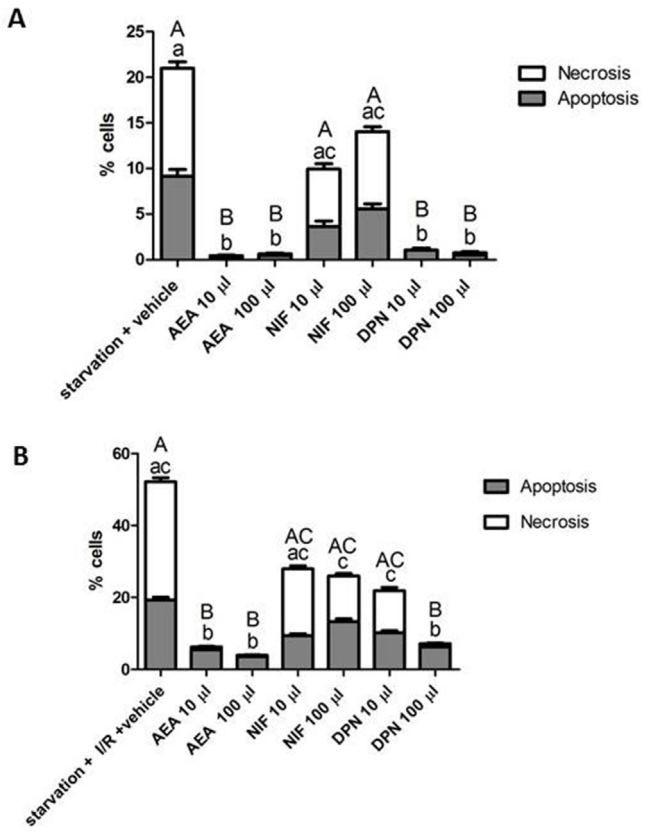

Fig. 7. Groups are: starvation + vehicle (in PBS + 1% dimethyl sulfoxide, DMSO) starvation + I/R + vehicle (in PBS + 1% dimethyl sulfoxide, DMSO + induction hypoxia and reoxygenation), AEA (anandamide at different concentrations), NIF (nifedipine at different concentrations), DPN (3,5-dipalmitoyl-nifedipine at different concentrations). The percentage of viable, apoptotic and necrotic H9c2 cells immediately after treatment of hypoxia and nutrient deprivation with 10 μM and 100 μM of anandamide (AEA, NIF and DPN from the visual field captured by a fluorescence microscope). Data are percentage of cells ± standard error. Different letters indicate significant differences compared to the control, with differences for necrotic cells indicated by uppercase letters and lowercase letters for apoptotic cells ($p < 0.05$).

pancreatic β-cells from the endoplasmic reticulum stress induced by glucose, decreasing the number of cells that underwent apoptosis and enhancing cell viability [37]. Evidence shows that although NIF has vasodilating action under normoxic conditions, it does not act as a vasodilator under hypoxic conditions [38]. Herein, this situation was confirmed, when NIF had no effect on cell viability during the induction of ischemia conditions. Together, these findings suggest that the cytoprotective action of nifedipine depends on concentration, cell type, and normoxic or hypoxic conditions.

Another substance that has shown a significant cardioprotective effect in a model of ischemia/reperfusion in rats is AEA, which decreased the incidence of ventricular arrhythmias and reduced infarct size through the activation of the cannabinoid receptor CB2 [39]. Additionally, antioxidant effects have been identified in the heart tissue of rats subjected to I/R compared with rats also subjected to I/R that did not express CB2 receptors [40]. AEA (100 μM) is also able to suppress calcium overload during I/R in cardiac myocytes, thus lowering the production of ROS [41]. Thus, AEA administered at concentrations of 10 μM and 100 μM decreased reactive oxygen species by 30% and 36.30%, respectively, in cardiomyoblasts subjected to I/R, proving the antioxidant action of this molecule.

The protective effects of AEA have also been associated with cytoprotective effects in toxicity models in vitro [42]. The therapeutic use of cannabinoids for multiple sclerosis, Parkinson's disease, and cardiac or cerebral ischemia has been suggested to improve cell viability [43,44]. This was also demonstrated by our results, because AEA at both concentrations increased cell viability in cardiomyoblasts subjected to I/R by 185.2% and 209%, respectively, compared with the starvation condition. The analysis of the death of cardiomyoblasts subjected to I/R treated with AEA (at both concentrations) and treatment with hybrid DPN (at a concentration of 100 μM) showed reduced values of apoptosis and necrosis.

Fatty acids such as palmitic acid (e.g., those involved in the induction of apoptosis in different cell types) can modulate the molecular pathways involved in apoptosis, including those in neonatal cardiomyocytes [45]. The process of adding unsaturated or saturated fatty acids to modify, add to, or enhance the effects of drugs has been reported in the literature; the time required to add this fatty acid can also influence the effect that is attributed to the molecule [46,47].

The search continues for new strategies to treat cardiac disorders; for example, multifunctional drugs that combine two entities, such as a drug and another molecule (e.g., DPN) create a new chemical structure that is able to modulate biological processes with completely new activity or multiple, combined activities [29]. Previously, analogues of dihydropyrimidinone hybrids with fatty acids (DHPM-fatty acids) have already been described as having an antiproliferative effect on glioma cells [18]; among such hybrids, the analog derivatives of palmitic and oleic acid have become the most promising drugs in treating this type of tumor [48].

According to literature, NIF affects cultured endothelial cells, however, these cells do not express L-type calcium channels, demonstrating that the effects of NIF are not restricted to these channels [49]. H9c2 cells only express L-type calcium channels to differentiate, and this condition did not occur during this experiment.

In the H9c2 line, joint administration of the omega-3 polyunsaturated fatty acid eicosapentaenoic acid (EPA) and palmitic acid can modulate the expression of the LC3 gene involved in the autophagosome formation response and promote a protective effect against apoptosis induced by palmitic acid [45]. The results herein demonstrated that when administered at a concentration of 100 μM just prior to the induction of ischemia and reperfusion, the new molecule, DPN, improved cell viability and decreased cell death from necrosis and apoptosis in cardiomyoblasts. In addition, DPN decreased the production of reactive oxygen species caused by hypoxia and reoxygenation, indicating that this molecule exerts an antioxidant effect, thus improving results when compared to treatment with NIF.

The scavenger characteristic of NIF and DPN was tested in in vitro assays; DPN and NIF could not stabilize the DPPH radical, a possible explanation for this lack of effect would be a low reactivity with this moiety [46]. In the other two trials using ABTS and FRAP, the ability of these molecules to be donors and/or share proton and chelate ferric ion was demonstrated. When compared with the IC_{50} of the ABTS test, the IC_{50} of DPN was 10 times smaller than that of NIF. When comparing the results of DPN with that of a classic antioxidant ascorbic acid through the same ABTS test [47], our hybrid molecule presented IC_{50} values less than twice that of ascorbate.

Benzie and Tomlinson [8] performed the FRAP test on various anti-hypertensive drugs. Among them, captopril presented iron-reducing power close to but lower than that of ascorbic acid. The lower values of IC_{50} for DPN demonstrate that our construction with two palmitic acid chains increased the scavenging power of DPN in vitro. This may be related to the effect of this in vivo molecule to significantly reduce reactive oxygen species in ROS

more efficiently than NIF. Thus, the construction of a hybrid molecule (NIF) with two palmitic acid molecules has the potential to be a drug offering more benefits in the treatment of hypertension due to potentiation of the antioxidant effect of the parent drug, thereby mitigating and preventing the damage caused by I/R.

These results are important because there is evidence that the process of joining molecules to fatty amides enhances the known effects of the molecule, likely by increasing the permeability of cells to these molecules [18]. This result increases the antioxidant effect and can also aid in the treatment of other diseases, because the ingestion of drugs with increased antioxidant effects can protect against cardiovascular disease [48].

However, it is important to discuss the antioxidant therapy in the context of cardioprotection considering the well-known ambiguous action of ROS. Recent reviews from [5,50] point out that ROS may have a good or bad effect on the I/R condition depending on the environment. As a good factor, ROS can promote redox signaling, a set of reversible reactions of oxidation of a signaling molecule by a reactive species, something like on-off signaling to phosphorylation [5]. Such benefits from ROS redox signaling are reached by pre-conditioning myocardium to ischemia and reperfusion injuries: for example, ROS generation by pharmacological pre-conditioning, considering that antioxidant therapy for cardiovascular disease over three decades has been reviewed by Pagliaro & Pena [5] and was found to fail to demonstrate cardiac protection in large clinical trials, from which the authors conclude that the absence of oxidative stress is not an effective means to mitigate I/R damage. They suggest that studies must find site- time- specific inhibition to ROS I/R injuries without affecting the redox-sensitive pathways for signaling cell survival [5]. Another important scenario of studies on myocardial I/R injuries is that proposed by Pagliaro & Pena [50] with a model of hypertrophic cardiomyoblasts, that considers the hypertrophy as a key target to the I/R therapy.

Thus, the novel hybrid molecule 3,5-DPN may act as pharmacological pre-conditioning for cardiovascular protection against I/R injuries by decreasing ROS in cardiomyoblasts subjected to I/R conditions and was also found to improve cell viability by 138.7% in a dose-dependent manner.

5. Conclusion

All cardiovascular diseases trigger the death of heart cells through apoptosis or necrosis; to improve this condition, interventions must be used to decrease the damage/impact of heart disease, such as related to ischemia and reperfusion. The new molecule synthesized 3,5-dipalmitoyl-nifedipine decreased ROS and improved viability in cardiomyoblasts subjected to ischemia and reperfusion condition. The results demonstrate that the hybridization procedure of two chains of palmitic acid to a nifedipine molecule assigns a greater cardioprotective effect to the molecule, probably due to an enhanced power scavenger and ferric ion reduction capability, thereby reducing the oxidative damage caused by ischemia and reperfusion in a cardiomyoblast culture. Future studies should evaluate the mechanism of action of the new molecule, especially to ensure that the translation to clinical application may be effective in cardiac injury protection especially in the heart hypertrophic condition.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biopha.2017.05.091.

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