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Cardioprotective effects of iron chelator HAPI and ROS-activated boronate prochelator BHAPI against catecholamine-induced oxidative cellular injury

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Abstract

Catecholamines may undergo iron-promoted oxidation resulting in formation of reactive intermediates (aminochromes) capable of redox cycling and reactive oxygen species (ROS) formation. Both of them induce oxidative stress resulting in cellular damage and death. Iron chelation has been recently shown as a suitable tool of cardioprotection with considerable potential to protect cardiac cells against catecholamine-induced cardiotoxicity. However, prolonged exposure of cells to classical chelators may interfere with physiological iron homeostasis. Prochelators represent a more advanced approach to decrease oxidative injury by forming a chelating agent only under the disease-specific conditions associated with oxidative stress. Novel prochelator (lacking any iron chelating properties) BHAPI [(E)-N'-(1-(2-((4-(4,4,5,5-tetramethyl-1,2,3-dioxoborolan-2-yl)benzyl)oxy)phenyl)ethylidene) isonicotinohydrazide] is converted by ROS to active chelator HAPI with strong iron binding capacity that efficiently inhibits iron-catalyzed hydroxyl radical generation. Our results confirmed redox activity of oxidation products of catecholamines isoprenaline and epinephrine, that were able to activate BHAPI to HAPI that chelates iron ions inside H9c2 cardiomyoblasts. Both HAPI and BHAPI were able to efficiently protect the cells against intracellular ROS formation, depletion of reduced glutathione and toxicity induced by catecholamines and their oxidation products. Hence, both HAPI and BHAPI have shown considerable potential to protect cardiac cells by both inhibition of deleterious catecholamine oxidation to reactive intermediates and prevention of ROS-mediated cardiotoxicity.

Keywords

Catecholamines; Cardiotoxicity; Iron chelation; HAPI; Prochelator; BHAPI

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1. Introduction

Catecholamines (CA), released as hormones of the adrenal medulla and neurotransmitters of the sympathetic nerve system, participate in an organism's stress response as part of the diffuse sympathetic discharge provoked in emergency situations. They regulate metabolism and cardiovascular system functions, where they affect systemic circulation *via* direct effects on myocardial contraction, excitability and vascular lumen (Cohn et al. 1984; Dhalla et al. 2010). Although CA secretion is low in basal states and is reduced even further during sleep, it increases under various pathophysiological conditions (Ganong 2005). Excessive and/or prolonged increase of either systemic or myocardial CA levels can induce heart injury, as occurs in: pheochromocytoma, stress-induced (Takotsubo) cardiomyopathy, acute myocardial infarction, cardiac arrhythmias, sudden cardiac death, or congestive heart failure (Cohn et al. 1984; Dhalla et al. 2010; Golabchi and Sarrafzadegan 2011; Mann et al. 1992). It is now generally accepted that the pathogenesis of the CA-induced myocardial injury is multifactorial. Numerous previous studies suggested the role of excessive β -adrenoreceptor stimulation leading to activation of protein kinase A, resulting in the downstream phosphorylation of multiple Ca^{2+} -cycling proteins, including sarcolemmal L-type Ca^{2+} channels, phospholamban and sarcoplasmic reticulum ryanodine receptor Ca^{2+} release channels (RyR_2). Persistent activation of β -adrenoreceptors may also promote the activation of Ca/calmodulin-dependent protein kinase II which phosphorylates multiple protein targets, including voltage-gated Ca^{2+} channels, RyR_2 Ca^{2+} release channels, with resulting calcium overload of cardiomyocytes. (Costa et al. 2011; Haskova et al. 2011; Rathore et al. 1998; Rona et al. 1959). The pathophysiological events triggered by calcium overload can be considerably amplified by the oxidative stress. Indeed, pathological levels of CA are accompanied with reactive oxygen species (ROS) production. Monoaminoxidase-dependent oxidative deamination of catecholamines forms hydrogen peroxide (H_2O_2), which may be converted to the highly reactive hydroxyl radical ($\text{OH}\cdot$) through metal catalysis. Furthermore, activation of α_1 -adrenoreceptors by catecholamines induces the activation of NADPH oxidase, with ensuing generation of the superoxide anion radical ($\text{O}_2^{\cdot-}$) (Liaudet et al. 2014). Probably the main pathway through which CAs induce oxidative cellular injury is represented by CA catabolism, which includes spontaneous oxidation of CA. This oxidation is a two-electron process forming ortho-quinone derivatives, followed by cyclization into leucoaminochromes which are further oxidized into aminochromes (Behonick et al. 2001; Haskova et al. 2011; Liaudet et al. 2014; Remiao et al. 2001). CA oxidation occurs spontaneously at a low rate (autooxidation), but it is markedly accelerated by enzymatic catalysis (notably by xanthine oxidase, myeloperoxidase and cytochrome oxidase), in the presence of oxidants and free radicals such as $\text{O}_2^{\cdot-}$, and it may be aggravated with transient metal catalysis (Haskova et al. 2011; Liaudet et al. 2014; Remiao et al. 2001).

Iron (Fe) is the most abundant transition metal in living organisms (Halliwell and Gutteridge 2007). It participates in a wide variety of metabolic processes, including oxygen transport, DNA synthesis, and electron transport. However, Fe concentrations must be tightly regulated as it may induce tissue damage due to the formation of free radicals (Lieu et al. 2001). Both the importance and potential toxicity of Fe stem from its ability to readily serve as an electron donor and acceptor due its cycling between its ferrous (Fe^{2+}) and ferric (Fe^{3+})

oxidation states (Halliwell and Gutteridge 2007). The potentially harmful Fe is represented by a low-molecular-weight pool of weakly chelated Fe that rapidly passes through the cell (Kruszewski 2004). Under normal conditions only a minute quantity of the intracellular Fe is present in this low-molecular-weight pool (Voogd et al. 1992). Labile Fe pool is increased in states with systemic Fe overload (Kremastinos et al. 2010; Wood 2009), but it may also change locally, when cellular Fe homeostasis may be severely deregulated by reactive oxygen or nitrogen species (Mladenka et al. 2006). For example, high cellular levels of superoxide and peroxide species, as well as low pH, both of which occur during myocardial ischemia-reperfusion injury, cause Fe release from its storage proteins, thereby increasing the cytosolic pool of labile Fe and exacerbating the production of deleterious ROS (Reif 1992). Also CA may release Fe from ferritin into labile Fe pool (Allen et al. 1994).

Fe-chelating therapy has been used in Fe-overload syndromes, such as those caused by repeated blood transfusions in patients with β -thalassemia major, where it promotes body Fe excretion (Hatcher et al. 2009; Kalinowski and Richardson 2005; Olivieri and Brittenham 1997). In addition, there is an emerging role for Fe chelators in the treatment of diseases characterized by oxidative stress associated with a local release of Fe, including inflammatory disorders, post-ischemic reperfusion injury, intoxication by xenobiotics, cardiovascular diseases, atherosclerosis, neurodegenerative diseases and cancer (Galey 2001; Hatcher et al. 2009). However, classical powerful Fe chelators may not be suitable for long-term treatment of oxidative stress-associated conditions without systemic Fe overload due to chelator-induced deregulation of physiological Fe homeostasis (Galey 2001). Therefore, an “ideal” Fe chelator to be used in oxidative stress-related conditions should not affect Fe homeostasis, but rather “strategically” chelate Fe only in oxidative cellular environment (Galey et al. 1995). Recently, boronate-masked prochelators have been introduced and evaluated (Bureš et al. 2015). They are designed not to bind metal ions unless the protective mask is conditionally removed by ROS to reveal an active chelator capable of suppressing Fe-mediated hydroxyl radical generation (Charkoudian et al. 2006; Jansova et al. 2016; Kielar et al. 2012).

The prochelator examined in this study, BHAPI (Fig. 1), is derived from HAPI, a salicylaldehyde isonicotinoyl hydrazone (SIH) analogue with improved hydrolytic stability (Hruskova et al. 2011). The boronyl mask of BHAPI is connected *via* a self-immolative spacer that undergoes spontaneous elimination after reacting with H_2O_2 (Kielar et al. 2012). In this study, we examined (1) the ability of CA (naturally occurring epinephrine (EPI) and synthetic isoprenaline (ISO)) and products of their spontaneous oxidation to activate BHAPI to effective Fe chelator HAPI, (2) the abilities of HAPI and BHAPI to penetrate H9c2 cells and bind intracellular labile Fe, (3) the level of protection provided by HAPI and BHAPI to protect cardiac cells against the toxicity induced by exposure to CA and their oxidative products, (4) the reduction of intracellular ROS achieved by HAPI and BHAPI and (5) the inherent toxicities of HAPI and BHAPI against H9c2 rat cardiomyoblasts and isolated ventricular cardiomyocytes from neonatal rats.

2. Material and methods

Chemicals

The catecholamines (*R*)-4-(1-hydroxy-2-(methylamino)ethyl)benzene-1,2-diol (epinephrine; EPI, Fig. 1) and (*RS*)-4-(1-hydroxy-2-(isopropylamino)ethyl)benzene-1,2-diol (isoprenaline; ISO, Fig. 1); Fe salts including ferrous ammonium sulfate (FAS) and ferric ammonium citrate (FAC); and other chemicals (*e.g.* constituents of various buffers) were purchased from Sigma-Aldrich (Germany) or Penta (Czech Republic) and were of the highest available pharmaceutical or analytical grade. The Fe chelator (*E*)-*N'*-(1-(2-hydroxyphenyl) ethyliden) isonicotinohydrazide (HAPI, Fig. 1) and prochelator (*E*)-*N'*-(1-(2-((4-(4,4,5,5-tetramethyl-1,2,3-dioxoborolan-2-yl)benzyl)oxy)phenyl)ethylidene) isonicotinohydrazide (BHAPI, Fig. 1) were synthesized as described previously (Hruskova et al. 2011; Kielar et al. 2012) and their identities and purities were confirmed with elemental analysis, ^1H and ^{13}C NMR, and IR spectroscopy. The chemicals and solutions used for cellular cultivation (*e.g.* cell-culture media, sera, *etc.*) were purchased from Sigma-Aldrich (Germany) or Lonza Group (Switzerland).

Overview of experimental protocols

All experiments were based on the ability of CA to undergo spontaneous oxidation, which is completed within 24 h (Haskova et al. 2011). There were five different protocols for the study of toxic and protective properties of compounds under investigation (Fig. 2). Briefly, working solutions of CA (ISO or EPI) used in all experiments were either freshly prepared or 24hpreoxidized (*i.e.* left spontaneously oxidize; oxCA) at 37 °C in the appropriate medium or buffer specified for each of the method (see below). The peroxidation step took place in absence of any cells. The Fe chelator or prochelator was added into the solution with CA either at the beginning of the experiment itself or at the beginning of 24h preoxidation of CA.

Ferric thiocyanate assay for assessment of redox-active intermediates in solution

The presence of redox-active intermediates formed during spontaneous oxidation of CA was assessed according to Seo *et al.* (Seo et al. 2004) *via* oxidation of ferrous ions to ferric ions and their consequent reaction with thiocyanate yielding reddish brown complex:

CA were incubated in serum-free cell-culture medium at 37 °C up to 48 h. Samples (100 µL) taken at times 0, 6, 12, 24, 30, 36 and 48 h during incubation were acidified with trichloroacetic acid and filled with water to volume of 1 mL. Furthermore, 200 µL of 10 µM FAS and 100 µL of 2.5 M K(SCN) were added. After 10 min, the light absorption (optical density) of formed ferric thiocyanate was measured using a microplate spectrophotometer Tecan Infinite 200 M (Tecan Austria, Austria) at $\lambda = 480$ nm. The concentration of redox-active intermediates was calculated from calibration curve of H_2O_2 and expressed as equivalents of H_2O_2 .

High-performance liquid chromatography (HPLC) analysis of prochelator activation

The HPLC determinations of prochelator BHAPI activation to HAPI by parent or oxidized EPI were performed in ADS buffer (116 mM NaCl, 5.3 mM KCl, 1.2 mM MgSO₄, 1.13 mM NaH₂PO₄, 5 mM glucose, 1 mM CaCl₂, 20 mM HEPES; pH 7.4) or in serum-free cell-culture medium – either without cells or with H9c2 cells.

The analysis was performed using an HPLC Prominence System (a DGU-20A3 degasser, two LC-20AD pumps, a SIL-20AC autosampler, a CTO-20AC column oven, a photodiode array detector and a CBM 20A communication module; Shimadzu, Japan). The column oven was set at 25 °C and the autosampler at 5 °C to prevent artificial degradation of samples.

Separation of BHAPI and HAPI was reached on an Ascentis C18 column (100×3 mm, 3 µm) protected by a guard column (Ascentis C18 20×3 mm, 5 µm) both from (Sigma-Aldrich, Germany). The mobile phase was composed of 15 mM NaH₂PO₄ (pH not adjusted) and acetonitrile (ACN) in gradient mode: 0-4 min (35-45 % of ACN), 4.01-10 min (45-50 % of ACN), 10.01-15 min (35 % of ACN). A flow rate of 0.25 mL/min and an injection volume of 30 µL were used. The quantification was made at $\lambda = 270$ nm and $\lambda = 283$ nm for BHAPI and HAPI, respectively. The linearity of the method was evaluated in the range of 1-100 µM; selectivity was confirmed by the analysis of blank samples supported by the calculation of the peak purity index using LC solution software (version 1.21 SP1, Shimadzu). HAPI was detected at 8.4 min and two isomers of BHAPI at 6.3 min and 7.4 min in all media. HPLC analyses were performed after 0, 1, 3, 6, 9, 12, and 24h or after 0, 15, 45, 105, 210 and 390min incubation of tested compounds (alone or in combinations) and their concentration changes were followed in time.

Cell culture

The H9c2 cell line derived from embryonic BD1X rat heart tissue (Kimes and Brandt 1976) was obtained from the American Type Culture Collection (ATCC; VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Lonza, Switzerland) supplemented with 10 % heat-inactivated fetal bovine serum (FBS; Lonza, Switzerland), 1 % penicillin/streptomycin solution (Lonza, Switzerland), and 10 mM HEPES buffer (pH 7.4; Sigma Aldrich, Germany). Cell cultivation was held in 75 cm² tissue culture flasks from Techno Plastic Products AG (TPP; Switzerland) at 37 °C in a humidified atmosphere of 5 % CO₂ in air. Cells were subcultured twice in a week when they reached approximately 90 % confluence (*i.e.* every 3rd - 4th day).

For experiments, cells were seeded into appropriate microplates or Petri dishes (TPP; Switzerland) at given cellular density. 24 h prior to all cellular experiments, the medium was changed for serum-free cell-culture medium (pyruvate-free DMEM (Sigma Aldrich, Germany) supplemented with 1 % penicillin/streptomycin solution and 10 mM HEPES buffer). Serum deprivation was used to stop cellular proliferation to mimic the situation in post-mitotic cardiomyocytes (Simunek et al. 2008). Pyruvate was omitted because it is an antioxidant and may interfere with ROS-related toxicity. The lipophilic compounds HAPI and BHAPI were dissolved in dimethyl sulfoxide (DMSO; Sigma Aldrich, Germany),

yielding a final concentration of 0.1 % in all experimental groups. At this concentration, DMSO had no effect on cellular viability.

Primary cultures of neonatal ventricular cardiomyocytes (NVCM) were prepared from 1-3 day old Wistar rats. All the procedures have been supervised and approved by the Ethical Committee of the Faculty of Pharmacy, Charles University in Prague. Briefly, neonatal hearts were minced in ADS buffer (1.2 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 116 mM NaCl; 5.3 mM KCl; 1.13 mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$; 20 mM HEPES) on ice, digested at 37°C with type II collagenase (Invitrogen, U.S.A.). After pre-plating on 150 mm Petri dishes for approximately 20 hearts to minimize non-myocyte contamination, cells were plated on gelatin-coated 24-well plates at a density of 40,000 cells per well. NVCM were cultured at 37°C and 5 % CO_2 in the DMEM/F12 medium supplemented with 10 % horse serum (HS; Lonza, Belgium), 4 mM pyruvate, 5 % FBS and 1% penicillin/streptomycin. Newly isolated NVCMs were left for 40 h to attach properly and form a culture of spontaneously beating cardiomyocytes. Then the medium was changed for 24 h to DMEM/F12 supplemented with 4 mM pyruvate, 5 % FBS and 1 % penicillin/streptomycin. For the experiments the medium was changed to serum- and pyruvate-free DMEM/F12 with 1 % penicillin/streptomycin. The cells were maintained in this medium until the end of experiments.

Calcein-AM assay for assessment of compounds' ability to permeate cell membrane and access to the labile Fe pool

The experiments were performed according to Glickstein *et al.* (Glickstein et al. 2006) with slight modifications. In brief, H9c2 cells seeded in 96-well plates at a density of 10,000 cells per well were loaded with Fe ions using the Fe donor, 100 μM FAC 24 h prior to the experiment. To avoid potential interference (especially with regard to various trace elements), the medium was discarded and cells were washed with the ADS buffer. After that, cells were loaded with a 1 μM calcein green acetoxymethyl ester (Calcein-AM; Molecular Probes, OR, USA) for 30 min at 37 °C and then washed. Calcein-AM is cell-permeable probe, to which the intracellular esterases cleave off the acetoxymethyl groups to form the cell membrane-impermeable compound, calcein, whose fluorescence is quenched by complexation with Fe ions. Intracellular green fluorescence ($\lambda_{\text{ex}} = 488 \text{ nm}$, $\lambda_{\text{em}} = 530 \text{ nm}$) was followed as a function of time: 1 min before and 10 min after the addition of compounds under investigation (alone or in combinations) using a microplate spectrophotometer Tecan Infinite 200 M.

Neutral red uptake assay for assessment of compounds' cytotoxicity

Cellular viability was determined using the assay based on the ability of viable cells to incorporate neutral red (NR; Sigma, Germany). This assay is based on the accumulation of NR in the lysosomes of viable cells (Repetto et al. 2008). H9c2 cells seeded in a 96-well plates at a density of 10,000 cells per well were incubated with compounds under investigation (alone or in combinations) for 24 or 72 h. Half of the medium volume from each well was removed at the end of incubation, and the same volume of medium with NR was added, yielding a final concentration of 40 $\mu\text{g/mL}$. After 3 h at 37 °C, the supernatant was discarded, and the cells were fixed in 0.5 % formaldehyde supplemented with 1 % CaCl_2 for 15 min. The cells were then washed twice with phosphate buffered saline and

solubilized with 1 % acetic acid in 50 % ethanol for 30 min of continuous agitation; thereby the accumulated NR was released into the extracellular fluid. The light absorption (optical density) of released NR was measured using a microplate spectrophotometer Tecan Infinite 200 M at $\lambda = 540$ nm. The viability of experimental groups was expressed as a percentage of the untreated control (100 %).

Determination of lactate dehydrogenase release to assess compounds' cytotoxicity and protective property

NVCMs were incubated at 37 °C with the tested agents either alone or in combination with EPI (700 μ M). The activity of lactate dehydrogenase (LDH) released from cardiomyocytes was determined in cell culture media as a standard marker of cytotoxicity and cellular breakdown using a well-established spectrophotometric method; in our previous studies, this method correlated well with release of cardio-specific troponins T and I (Adamcova et al. 2007). The activity of LDH released from cardiomyocytes was expressed as the percentage of total cellular LDH as measured following cell lysis for 15 min (0.1 M potassium phosphate, 1% Triton X-100, 1 mM DTT [(–)-1,4-dithio-L-threitol], 2 mM EDTA, pH 7.8) at room temperature. All the samples were frozen immediately and kept in –80°C prior to measurement. Activity of LDH was assayed in Tris–HCl buffer (100 mM, pH 8.9) containing 35 mM of lactic acid and 5 mM of NAD⁺. The rate of NAD⁺ reduction was monitored spectrophotometrically at 340 nm for 2 min. The slope of the linear region and molar absorption coefficient $\epsilon = 6.22 \cdot 10^3$ M/cm was used for calculation of LDH activity and the data was expressed as the percentage of total LDH amount.

Epifluorescence microscopy for assessment of cellular morphology changes

Changes in morphology of H9c2 cells were observed and imaged using an inverted epifluorescence microscope Nikon Eclipse TS100 with 10-40x air objectives (Nikon, Japan) equipped with a digital camera 1300Q (VDS Vosskühler, Germany) and the software NIS-Elements AR 3.10 (Laboratory Imaging, Czech Republic). Furthermore, the cellular viability was visualised using nuclei staining with Hoechst 33342 (Molecular Probes, OR, U.S.A.) and propidium iodide (PI; Molecular Probes, OR, U.S.A.), which are well-established and sensitive probes to determine apoptosis and necrosis: Hoechst 33342 is a blue-fluorescent probe ($\lambda_{\text{ex}} = 360$ nm, $\lambda_{\text{em}} = 460$ nm) staining all nuclei. In apoptotic cells, chromatin condensation occurs and apoptotic cells can thus be identified as those with condensed and more intensely stained chromatin. The red-fluorescent ($\lambda_{\text{ex}} = 560$ nm, $\lambda_{\text{em}} = 630$ nm) DNA-binding dye, PI, is unable to cross the plasma membrane of living cells, but readily enters necrotic (or late-stage apoptotic) cells and stains their nuclei red. H9c2 cells seeded in 12-well plates at a density of 75,000 cells per well were incubated with compounds under investigation (alone or in combinations) for 24 h. After that, cells were stained with 10 μ g/mL Hoechst 33342 and 1 μ g/mL PI for 20 min at room temperature.

Epifluorescence microscopy for determination of mitochondrial inner membrane potential

Mitochondrial activity was assessed in NVCMs using the JC1 probe (5,5',6,6'-tetrachlor-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide; Molecular Probes/Invitrogen, Czech Republic). JC1 becomes non-specifically accumulated in cellular cytosol as a green fluorescent monomer ($\lambda_{\text{ex}} = 480$ nm; $\lambda_{\text{em}} = 535$ nm). In metabolically-active

mitochondria with polarized inner membrane, JC1 monomers flock into red fluorescent J-aggregates ($\lambda_{\text{ex}} = 560 \text{ nm}$; $\lambda_{\text{em}} = 630 \text{ nm}$). Cells were seeded on 24-well plates and incubated for 24 h with tested compounds and then loaded with $3 \mu\text{M}$ JC1 for 15 min at 37°C after which the medium was replaced with PBS. Photomicrographs were obtained using the epifluorescence inverted microscope Eclipse TS100 (Nikon, Japan) equipped with digital cooled camera (1300Q, VDS Vosskühler, Germany) and software NIS-Elements AR 3.10 (Laboratory Imaging, Czech Republic).

2',7'-Dichlorodihydrofluorescein diacetate assay for determination of cellular reactive oxygen species formation

To assess cellular ROS generation, the measurement of 2',7'-dichlorodihydrofluorescein diacetate ($\text{H}_2\text{DCF-DA}$; Molecular Probes, OR, U.S.A.) fluorescence intensity was used. This originally non-fluorescent reagent diffuses passively through the cellular membranes into the cell, where acetate groups are cleaved by intracellular esterases and subsequently oxidized by ROS formed within the cell (particularly by hydroxyl radicals; $\bullet\text{OH}$) to green-fluorescent 2',7'-dichlorofluorescein (DCF; $\lambda_{\text{ex}} = 485 \text{ nm}$, $\lambda_{\text{em}} = 525 \text{ nm}$); the fluorescence intensity is proportional to the $\bullet\text{OH}$ concentration, leading to a quantitative measurement of intracellular $\bullet\text{OH}$.

H9c2 cells seeded in a 96-well plate at a density of 10,000 cells per well were washed twice with ADS buffer and loaded with $10 \mu\text{M}$ $\text{H}_2\text{DCF-DA}$ in ADS buffer. After 60 min incubation, the loading buffer was discarded, and the cells were washed twice with ADS buffer and exposed to compounds under investigation (alone or in combinations) prepared in ADS buffer. All solutions were pre-warmed to 37°C . Fluorescence intensity was measured for 30 min at 37°C using a microplate spectrophotometer Tecan Infinite 200 M. ROS formation in the experimental groups was expressed as a percentage of the untreated control (100 %).

Determination of total and oxidized glutathione contents

H9c2 cells seeded in a 100 cm Petri dish at a density of 1.2 million cells per dish were incubated with HAPI or BHAPI ($100 \mu\text{M}$) alone or in combination with oxEPI ($60 \mu\text{M}$) for 24 hours. Cells were then rinsed twice with pre-cooled PBS, scraped off and centrifuged (10 min, $700 \times g$, 4°C). The supernatant was aspirated completely, $250 \mu\text{l}$ of 4°C cold sulphosalicylic acid (Sigma, Germany) was added and the samples were stored in -80°C . Prior to analysis, the pellets were resuspended and sonicated on ice for 10 s (Bandelin Sonoplus, Bandelin Instruments, Germany). Ten microliters of the cell lysate was taken for determination of protein content, whereas the rest of the lysate was centrifuged for 15 min ($14,000 \times g$, 4°C), and the supernatants were used for glutathione determination according to (Tietze 1969) using the enzymatic recycling method with 5,5'-dithio-bis (2-nitrobenzoic acid) (Sigma, Germany) and glutathione reductase (Sigma, Germany) in a microplate format using plate reader TECAN Infinite M200 (Tecan, Austria). Oxidized glutathione was determined after masking the reduced glutathione with 2-vinylpyridine (Sigma, Germany). The rate of 2-nitro-5-mercaptobenzoic acid formation was recorded at 405 nm for 2 min, and the slope of linear region of the reaction in individual samples was compared to the slopes of appropriate standards. The concentration of total or oxidized glutathione was calculated

using the method of linear regression, and the results were corrected for protein content determined spectrophotometrically using bicinchoninic acid kit with bovine serum albumin as a standard according to the manufacturer's protocol (QuantiPro™ BCA, Sigma, Germany).

Data analysis

In this study, the statistical software SigmaStat for Windows 3.5 (SPSS, CA, USA) was used. Data are presented as mean $\pm$ *S.D.* (standard deviation). Statistical significance was assessed using one-way or two-way ANOVA with a Bonferroni post hoc test (comparisons of multiple groups against corresponding controls). Results were considered to be statistically significant when $p < 0.05$. The biological half-lives of tested compounds ($t_{1/2}$ values); the concentrations of compounds inducing 50 % loss of cellular viability (TC_{50} values); and the concentrations leading to 50 % protection of cellular viability from toxicity induced by CA (EC_{50} values) were calculated using the CalcuSyn 2.0 software (Biosoft, U.K.). The graphs were created using GraphPad Prism 6.04 for Windows (GraphPad Software, CA, U.S.A.).

3. Results

Assessment of redox-active intermediates formed from CA

Oxidation of CA (ISO and EPI, 10 – 300 μ M) to redox-active intermediates was estimated with a standard method used for H_2O_2 determination in solution (Seo et al. 2004). This spectrophotometric assay is based on formation of ferric thiocyanate ($\lambda = 480$ nm) and revealed continuous formation of redox-active intermediates from both CA during their 48h incubation (Fig. 3). The levels of ROS formed were expressed as equivalents of H_2O_2 (which was used for calibration of this assay) and, interestingly, they reached values that were equimolar to that of the parent CA assayed.

Assessment of HAPI and BHAPI stabilities and CA-induced BHAPI activation

HPLC analysis was used to monitor the stabilities of HAPI and BHAPI and activation of BHAPI to HAPI (all 100 μ M) during their 24h incubation alone or in combination with EPI (either freshly-prepared or 24h-preoxidized). Without EPI, HAPI and BHAPI were relatively stable, their detectable concentration decreased by only approximately 20 % of their initial amount at the end of incubation period (Fig. 4A, D, G). In the case of BHAPI in ADS buffer, there was almost no loss of concentration, suggesting its higher stability as compared to HAPI (Fig. 4A).

Freshly-prepared EPI caused significant decrease in BHAPI concentration (Fig. 4B, E, H), particularly in cell-culture medium (either with or without cells). The activation of BHAPI was associated with an increase of corresponding chelator HAPI. This process was faster in cell-culture medium without cells ($t_{1/2} = 3.0$ h) than with cells ($t_{1/2} = 4.8$ h), probably due to cellular antioxidant systems partially detoxifying the ROS formed upon EPI oxidation. Similar results were observed in experiments where 24h-preoxidized EPI was used for BHAPI activation. Complete decomposition of BHAPI to HAPI occurred and this was again

faster in experiments in cell-culture medium without cells than with H9c2 cardiomyoblasts ($t_{1/2} = 2.9$ h vs. $t_{1/2} = 4.4$ h, respectively).

Determination of compounds' ability to permeate cell membrane and access to the labile Fe pool

The calcein-AM assay was used to compare the ability of studied compounds to access the labile Fe pool in H9c2 cells and chelate free or loosely bound Fe ions. The cell permeation process and subsequent intracellular Fe chelation was monitored during a 10-min incubation of cells with Fe chelator HAPI or prochelator BHAPI (100 μ M) alone or in combination with 24h-preoxidized CA (oxISO, oxEPI, both 60 μ M). The fluorescence increase was caused by the removal of Fe from its intracellular trapped complex with calcein and the dequenching of its fluorescence.

As seen in Fig. 5, BHAPI was not able to bind Fe from the complex with calcein and thus no significant increase in fluorescence was observed. No increase in fluorescence was also seen when the cells were incubated with oxISO or oxEPI. On the other hand, BHAPI activated with both oxISO and oxEPI showed a significant increase in fluorescence intensity of free calcein and after 10 min. it reached chelating activity of HAPI.

Cellular toxicity studies using the H9c2 cardiomyoblast cell line

The H9c2 rat cardiomyoblast-derived cell line was used for assessment of the potential cardioprotective effects of HAPI and BHAPI against the toxicity induced by CA (ISO or EPI) and their oxidation products (oxISO and oxEPI). Cytotoxicity studies were based on a neutral red uptake assay and followed the multiple protocols shown in Fig. 2.

Firstly, the protective effects of HAPI and BHAPI (1–600 μ M) were assayed in an experimental setup in which the tested compound was co-incubated for 24 h with freshly-prepared EPI or ISO solutions at a concentration of 300 μ M; these concentrations and incubation times of CA were previously shown to induce complete loss of cellular viability (Haskova et al. 2011). As seen in Fig. 6A-B, both HAPI and BHAPI were able to partially but significantly protect the cells in a concentration-dependent manner, although in the case of BHAPI there was a decrease in cellular viability at its highest concentration (300–600 μ M) probably due to its inherent toxicity.

The protective potential of HAPI and BHAPI to protect against the toxicities induced by oxISO or oxEPI (both 60 μ M, based on our previous study (Haskova et al. 2011)) was then assayed by two protocols (Fig. 2 D & E). In the first protocol, CA was pre-oxidized for 24 h prior to addition to cells with the test compound (Fig. 6C-D), while in the second protocol the test compounds were pre-incubated with CA for the 24 h oxidation step prior to addition to cells (Fig. 6E-F). BHAPI was able to significantly protect H9c2 cells and this effect was comparable to the protection provided by HAPI. They both preserved approximately 50 % of cellular viability in all tested concentrations with the exception of the highest concentration (600 μ M), where BHAPI showed decrease in cardioprotective potential, again probably due to its inherent toxicity.

The protective potential of HAPI and BHAPI against CA cardiotoxicity was also verified using epifluorescence microscopy, where 24h incubation of cells with 60 μM oxEPI induced peripheral membrane blebbing, which was followed by the loss of the spindle-like cell shape and rounding up of cells. Furthermore, severe nuclear condensation occurred and eventually a complete loss of cellular viability was followed by the formation of cell debris. Furthermore, the oxEPI-treated cells were stained by cell membrane-impermeable red PI, indicative of cellular necrosis. Both HAPI and BHAPI (100 μM) were able to distinctively protect cellular morphology of H9c2 cells against this oxEPI-induced damage (Fig. 7).

Inherent cytotoxicities of HAPI and BHAPI (1–600 μM) following their 24 h or 72 h incubation with H9c2 cells are shown in Fig. 8. Both compounds induced dose-dependent decrease in cellular viability, when significant reductions of cellular viability were observed at concentration of 300 μM of HAPI ($\text{TC}_{50} > 600 \mu\text{M}$) and BHAPI ($\text{TC}_{50} = 363 \mu\text{M}$) after 24 h. Although BHAPI was more toxic in acute experiments (24 h), upon 72 h incubation, it showed lower inherent toxicity ($\text{TC}_{50} = 37 \mu\text{M}$) compared to parent chelator HAPI ($\text{TC}_{50} = 16 \mu\text{M}$).

Cardiotoxicity studies using primary cultures of neonatal rat cardiomyocytes

Epifluorescence microscopy with the JC1 probe was used to assess the inner mitochondrial membrane potential (Ψ_m). NVCMs were incubated for 24 h with HAPI or BHAPI (30 μM) alone or in combination with 24h-preoxidized EPI (700 μM). As seen in Fig. 9A, the chelator HAPI and prochelator BHAPI induced almost no reduction of red fluorescence intensity, indicating very slow and transient loss of Ψ_m . The oxEPI-treated cells displayed a distinct red to green fluorescence transition indicating almost complete Ψ_m dissipation. Whereas HAPI and BHAPI markedly reduced the oxEPI-induced Ψ_m loss.

These results were confirmed by determination of inherent toxicity and protective effect of HAPI and BHAPI using assessment of lactate dehydrogenase release. Inherent toxicities of these compounds (30, 100, 300 μM) after 24 h incubation with NVCM are shown in Fig. 9B, C. BHAPI induced significant and dose-dependent decrease in cellular viability. As in experiments with H9c2 cells, HAPI exhibited lower, although statistically significant toxicity in these acute experiments than prochelator BHAPI.

HAPI significantly preserved NVCM viability decreased by oxEPI in 30 and 100 μM concentrations. Prochelator BHAPI significantly protected NVCM cells against oxEPI-induced damage only in 30 μM concentration. At higher concentrations, no significant protective effect was observed probably due to its increasing inherent toxicity (Fig. 9D, E).

Determinations of intracellular ROS formation

The $\text{H}_2\text{DCF-DA}$ assay was used to compare the ability of studied compounds to prevent ROS generation in H9c2 cells. This ROS formation was measured at the end of 30-min incubation of cells with HAPI or BHAPI (10–600 μM) in combination with 24h-preoxidized CA (oxISO or oxEPI; 60 μM). This assay is based on the oxidation of intracellularly trapped non-fluorescent H_2DCF to fluorescent DCF. As seen in Fig. 10, both oxISO and oxEPI significantly induced ROS production inside H9c2 cells as compared to the untreated control group. Co-incubation with both HAPI and BHAPI decreased ROS formation; the

significantly effective concentrations of BHAPI were 30 μ M and 10 μ M in the case of HAPI.

Determination of total and oxidized glutathione

Cellular ability to adapt to oxidative stress was described using assessment of total and oxidized glutathione after 24 h incubation of H9c2 cells with tested compounds alone or in combination with oxEPI. As seen in Fig. 11A, 60 μ M oxEPI markedly decreased total glutathione content that was probably caused by its release from necrotic cells with disrupted cellular membranes. HAPI or BHAPI (100 μ M) did not affect the total glutathione content and were both able to prevent the depletion induced by oxEPI. The ratio between oxidized (GSSG) and total glutathione was significantly increased in oxEPI-treated cells indicating severe disruption of the cellular redox homeostasis (Fig. 11B). Whereas neither HAPI nor BHAPI affected the cellular GSSG content, they were both able to partially (and in the case of HAPI also significantly) diminish the GSSG increase caused by oxEPI (Fig. 11B).

4. Discussion

Apart from excessive β -adrenoreceptor stimulation and/or induction of calcium overload of cardiac cells, an increasing body of experimental evidence suggests that the pathophysiological mechanisms of CA-induced cardiac injury involve Fe-catalyzed formation of ROS (Costa et al. 2011; Dhalla et al. 2010). Spontaneous oxidation of CA results in formation of unstable *o*-quinones and subsequently aminochromes that can be further oxidized to other reactive compounds. These products of CA oxidation can promote the development of Haber-Weiss reactions and production of ROS, especially the highly reactive and toxic $\bullet$ OH (Bindoli et al. 1992). This has been also demonstrated in the present study. We observed that CA formed redox-active intermediates and that their concentrations, expressed as equivalents of H_2O_2 , reached comparable values as the concentrations of parent tested CA (Fig. 3).

We previously described promising cardioprotective properties of the Fe chelator SIH (Haskova et al. 2011). SIH belongs to the group of pyridoxal isonicotinoyl hydrazone analogs (Kalinowski and Richardson 2005). It is a tridentate, small and highly lipophilic Fe chelator that can be administrated orally. SIH is able to bind intracellular redox-active Fe and thus prevent the production of ROS (Bendova et al. 2010; Haskova et al. 2011; Horackova et al. 2000; Jansová et al. 2014; Simunek et al. 2005; Sterba et al. 2007). However, a serious disadvantage of SIH is represented by fast hydrolysis of its labile hydrazone bond (Buss et al. 2002; Kovariková et al. 2005; Kovariková et al. 2008). For this reason, its methyl ketone derivative, HAPI, was prepared with considerably enhanced hydrolytic stability (Hruskova et al. 2011). It has been already shown that HAPI was able to protect H9c2 cells against damage induced by H_2O_2 (Jansová et al. 2014) and *tert*-butylhydroperoxide (Hruskova et al. 2011). Results of the present study demonstrated that HAPI was able to slow down the CA-induced ROS formation (Fig. 10) and it significantly decreased the cardiotoxicity of the oxidation products of CA (Fig. 6, Fig. 9). We also demonstrated its good intracellular Fe chelating activity (Fig. 5) and low inherent toxicity of

HAPI following 24 h incubation ($TC_{50} > 600 \mu M$; Fig. 8). However, the toxicity of HAPI was markedly increased when the incubation period was prolonged to 72 h, apparently due to the Fe withdrawal.

Recent efforts are aimed at developing pro-drugs that avoid toxicity caused by Fe depletion. This strategy in principle allows targeted chelation therapy for pathological states not connected with systemic Fe overload, but for which local Fe lability contributes to cellular oxidative stress. In this study, we examined the prochelator BHAPI that has boron-containing mask, the removal of which is possible only under conditions of oxidative stress (Kielar et al. 2012). Its potential protective effect has already been mentioned in two previous studies, where BHAPI was able to significantly protect H9c2 cells against H_2O_2 -induced damage and ARPE cells against cellular injury caused by redox-active herbicide paraquat (Jansová et al. 2014; Kielar et al. 2012). Our results here showed that BHAPI significantly decreased the production of toxic intermediates from CA at $30 \mu M$ and higher concentrations (Fig. 10). We also verified significant cardioprotective properties of BHAPI against injury caused by 24hpreoxidized CA (ox EPI and oxISO). These effects were comparable with the protective efficiency of HAPI itself, with the exception of its $600 \mu M$ concentration, where the protective effect was lower (Fig. 6C, D, E, F), apparently due to its own toxicity. Although BHAPI possessed almost no toxicity up to its $100 \mu M$ concentration, in higher concentrations, its inherent toxicity increased and it was even higher than the toxicity of HAPI (Fig. 8A-B). On the other hand, BHAPI showed a better toxicity profile than HAPI in long-term (72 h) experiments (Fig. 8C-D). We also demonstrated that BHAPI could not bind Fe ions unless activated by ROS generated upon exposure to oxEPI or oxISO (Fig. 5), conditions for which it reached the chelating activity of HAPI. Its activation to HAPI was also confirmed by an HPLC method (Fig. 4). BHAPI, incubated with freshly-prepared or 24h-preoxidized EPI, was completely decomposed at the end of the experiments in DMEM medium with or without H9c2 cells and nearly equimolar amounts of HAPI were formed. A small loss of concentration of formed HAPI was probably associated with its own stability.

In conclusion, in the present study we confirmed that free redox-active cellular Fe plays an important role in CA conversion to oxidation products as well as their cardiotoxicity, which results in nonprogrammed cell death. We observed advantageous properties of both the Fe chelator HAPI and its derived prochelator BHAPI. Our experiments showed good stability of both compounds, especially of BHAPI, and confirmed ability of the prochelator to be activated under conditions of oxidative stress induced by oxidized CA to active chelator HAPI and subsequently to significantly reduce intracellular ROS generation and further oxidative stress propagation. This study suggests that research of novel Fe chelators and particularly Fe prochelators deserves further investigation and that both HAPI and BHAPI are good candidates for further *in vivo* study.

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Abbreviations

ACN	acetonitrile
BHAPI	(E)-N'-(1-(2-((4-(4,4,5,5-tetramethyl-1,2,3-dioxoborolan-2-yl)benzyl)oxy)phenyl)ethylidene)isonicotinohydrazide
CA	catecholamine
CHEL	iron chelator
DCF	2',7'-dichlorofluorescein
DMSO	dimethyl sulfoxide
EC₅₀ value	concentration that reduces the toxicity induced by catecholamines to 50 % of the viability of control cells (half-maximal effective concentration)
EPI	epinephrine
FAC	ferric ammonium citrate
FAS	ferrous ammonium sulphate
FBS	fetal bovine serum
Fe	iron
GSH	reduced glutathione
GSSG	oxidized glutathione
H₂DCF-DA	2',7'-dichlorodihydrofluorescein diacetate
HAPI	(E)-N'-(1-(2-hydroxyphenyl) ethylden)isonicotinohydrazide
ISO	isoprenaline
oxCA	24h-preoxidized catecholamine
oxEPI	24h-preoxidized epinephrine
oxISO	24h-preoxidized isoprenaline
PI	propidium iodide
proCHEL	iron prochelator
ROS	reactive oxygen species
SIH	(E)-N'-(2-hydroxybenzylidene) isonicotinohydrazide

t_{1/2} values	biological half-life of the compound under investigation
TC₅₀ values	concentration that induces 50% viability reduction compared to control cells (half-maximal toxic concentration)

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Highlights

- Fe prochelator BHAPI was examined for protection against catecholamine cardiotoxicity.
- BHAPI has no affinity for iron ions until it is activated to active chelator HAPI.
- Catecholamines, as a source of ROS, induced conversion of BHAPI into HAPI.
- BHAPI and HAPI decrease ROS formation induced by catecholamines.
- BHAPI and HAPI protect H9c2 cells against oxidative injury induced by catecholamines.

CATECHOLAMINE

-R:

-CH₃ = EPINEPHRINE

$$-\text{CH}_2(\text{CH}_3)_2 = \text{ISOPRENALINE}$$

AMINOCHROME

-R:

-CH₃ = ADRENOCHROME

$$-\text{CH}_2(\text{CH}_3)_2 = \text{ISOPRENOCHROME}$$

BHAPI

HAPI

Figure 1. Structures of the tested compounds

CA: (*R*)-4-(1-hydroxy-2-(methylamino)ethyl)benzene-1,2-diol (EPI) and (*RS*)-4-(1-hydroxy-2-(isopropylamino)ethyl)benzene-1,2-diol (ISO); aminochromes *i.e.* CA oxidation products: 3-hydroxy-1-methyl-2,3-dihydro-1H-indole-5,6-dione (adrenochrome) and 3-hydroxy-1-isopropyl-2,3-dihydro-1H-indole-5,6-dione (isoprenochrome); Fe prochelator: (*E*)-N'-(1-(2-((4,4,5,5-tetramethyl-1,2,3-dioxaborolan-2-yl)benzyl)oxy)phenyl)ethylidene) isonicotinohydrazide (BHAPI); Fe chelator: (*E*)-N'-(1-(2-hydroxyphenyl)ethyliden) isonicotinohydrazide (HAPI) and its complex with Fe(III) [Fe(HAPI)₂]⁻.

Figure 2. Overview of the protocols showing procedures of cellular studies

Working solutions of CA (ISO or EPI) used in all experiments were either (A-B) freshly-prepared or (C-E) 24hpreincubated (*i.e.* left to spontaneously oxidize; oxCA) at 37 °C. The Fe chelator or prochelator [(pro)CHEL] were added to solutions with catecholamine either (B, D) at the start of the cellular experiment itself or (E) at the beginning of 24h preincubation of CA.

Figure 3. Assessment of CA redox-active intermediates formation

Spontaneous oxidation of CA (EPI and ISO) in serum-free cell-culture medium was determined as the increase of redox-active H₂O₂ equivalents. Time course of CA redox-active intermediates formation during their 48h spontaneous oxidation. Data are presented as means ± S.D.; *n* = 4; Statistical significance (ANOVA, *p* < 0.05): * vs. t₀.

Figure 4. Process of BHAPI activation to HAPI

HPLC analyses of spontaneous degradation of BHAPI and HAPI and of effects of freshly-prepared or 24h-preoxidized EPI. BHAPI or HAPI were incubated for 24 h: (A, D, G) alone, (B, E, H) with EPI or (C, F, I) with oxEPI in (A, B, C) buffered solution (pH 7.4), (D, E, F) serum-free cell-culture medium or (G, H, I) serum-free cell-culture medium with H9c2 cells. Data are presented as means $\pm$ S.D.; $n = 4$; Statistical significance (ANOVA, $p < 0.05$): * vs. t_0 .

Figure 5. Intracellular Fe-chelating efficiency of Fe chelator HAPI and prochelator BHAPI

Effects of 24h-preoxidized CA isoprenaline (oxISO) or epinephrine (oxEPI) on prochelator BHAPI inside H9c2 cells were determined during 10 min with Calcein-AM assay. (A, C) Time course of BHAPI activation to effective chelator with (A) oxISO or (C) oxEPI. (B, D) Efficiency of prochelator activation to effective chelator with (B) oxISO or (D) oxEPI at time 10 min. Data are presented as means $\pm$ *S.D.*; *n* = 4; Statistical significance (ANOVA, *p* 0.05): * *vs.* control, # *vs.* corresponding Fe chelator.

Figure 6. Comparison of protective effect of HAPI and BHAPI against ISO- and EPI-induced toxicities towards H9c2 cardiomyoblast cell line

Cellular viabilities were determined by neutral red uptake assay and expressed as a percentage of the untreated control group. (A) HAPI or (B) BHAPI were added to freshly-prepared CA in serum-free cell-culture medium before the start of 24h cellular experiments; (C) HAPI or (D) BHAPI were added immediately before cellular experiments to CA preoxidized for 24 h in serum-free cell-culture medium; (E) HAPI or (F) BHAPI were preincubated for 24 h together with CA in serum-free cell-culture medium and then added to cells. Data are presented as means $\pm$ S.D.; $n = 4$; Statistical significance (ANOVA, $p < 0.05$): * vs. control group, # vs. corresponding CA group.

Figure 7. Cellular morphology and nuclear epifluorescence staining with Hoechst 33342 and PI H9c2 cardiomyoblasts were incubated for 24 h with (A) control medium, (B) 24h-preoxidized EPI (oxEPI) alone, or in combination with studied compound (C) HAPI or (D) BHAPI, when the compound was added to oxEPI immediately before cellular experiment. Scale bars represent 100 μm.

Figure 8. Comparison of cytotoxic effects of Fe chelator HAPI and prochelator BHAPI towards H9c2 cardiomyoblast cell line

Cellular viabilities were determined by neutral red uptake assay and expressed as a percentage of the untreated control group. H9c2 cells were incubated with increasing concentrations of tested compounds for 24h (A, B) or 72h (C, D) Data are presented as means $\pm$ *S.D.*; *n* = 4; Statistical significance (ANOVA, *p* < 0.05): * vs. control group.

Figure 9. Protective effects of HAPI and BHAPI against oxEPI-induced toxicities towards isolated rat neonatal ventricular cardiomyocytes (NVCM)

NVCM were incubated for 24 h with studied compounds or with their combination with 24h-preoxidized EPI (oxEPI; 700 μM). (A) Using epifluorescence microscopy, mitochondrial depolarization was assessed after loading with the JC-1 probe (red emission reflects mitochondrial inner membrane potential-dependent accumulation of probe dimers in actively respiring mitochondria, green fluorescence indicates monomers of the probe released into the cytoplasm after mitochondrial depolarization, lack of fluorescence reflects probe release from necrotic or late-stage apoptotic cells). Scale bars represent 50 μm. (B, C) Inherent toxicities of HAPI and BHAPI determined after 24 h incubation with NVCM by measurement of lactate dehydrogenase release. (D, E) Protective effects of HAPI and BHAPI against oxEPI-induced damage on NVCM assessed by measurement of lactate dehydrogenase release after 24 h incubation. Data are presented as means ± S.D.; n = 4; Statistical significance (ANOVA, $p < 0.05$): * vs. control group, # vs. oxEPI group.

Figure 10. Comparison of effects of HAPI and BHAPI on cellular oxidative stress induced by 24h-preoxidized CA (oxCA – EPI and ISO)

Intracellular ROS formation was determined by H₂DCF-DA assay following the 30min treatment of H9c2 cardiomyoblasts with combination of oxCA and studied compounds.

Induced intracellular fluorescence was expressed as a percentage of the group treated with oxCA alone. Cells were incubated with various concentrations of (A) HAPI or (B) BHAPI added immediately before cellular experiments to medium with oxCA (60 μM). Data are presented as means ± S.D.; *n* = 4; Statistical significance (ANOVA, *p* < 0.05): * vs. control group, # vs. corresponding CA group.

Figure 11. Effects of studied compounds on intracellular glutathione levels

H9c2 cells were incubated for 24 h with chelator HAPI and prochelator BHAPI (100 μ M) or with their combinations with oxEPI (60 μ M). (A) Total intracellular glutathione content; (B) The ratio of oxidized glutathione (GSSG) on total glutathione content. Data are presented as means $\pm$ S.D.; $n = 3$; statistical significance (ANOVA, $p < 0.05$): * vs. control group; # vs. oxEPI group.