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TOXICOKINETICS AND METABOLISM

Structural isomerization of synephrine influences its uptake and ensuing glutathione depletion in rat-isolated cardiomyocytes

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Abstract Synephrine is a natural compound, frequently added to ephedra-free dietary supplements for weight-loss, due to its effects as a nonspecific adrenergic agonist. Though only *p*-synephrine has been documented in plants, the presence of *m*-synephrine has also been reported in weight-loss products. The use of synephrine in dietary supplements was accompanied by reports of adverse effects, especially at the cardiovascular level. It is well known that the imbalance in cardiac glutathione levels can increase the risk of cardiomyopathy. The present work aimed to study the role of organic cation-mediated transport of *m*- and *p*-synephrine and the possibility that *p*- and *m*-synephrine induce intracellular changes in glutathione levels in calcium-tolerant freshly isolated cardiomyocytes from adult rat. After a 3 h incubation with 1 mM *p*- or *m*-synephrine, the intracellular content of synephrine was measured by gas chromatography/ion trap-mass spectrometry (GC/IT-MS); cell viability and intracellular glutathione levels were also determined. To evaluate the potential protective effects of antioxidants against the adverse effects elicited by *m*-synephrine, cells were pre-incubated for 30 min with Tiron (100 μM) or N-acetyl-cysteine (NAC) (1 mM). To assess the influence of α_1-adrenoceptors activation in glutathione depletion, a study with prazosin (100 nM) was also performed. The results obtained provide evidence that organic cation transporters OCT3 and OCT1 play a major role in *m*- and *p*-synephrine-mediated transport into the cardiomyocytes. The importance of these transporters seems similar for both isomers, although *p*-synephrine enters more into the cardiomyocytes. Furthermore, only *m*-synephrine induced intracellular total glutathione (GSHt) and reduced glutathione (GSH) depletion. NAC and Tiron were able to counteract the *m*-synephrine-induced GSH and GSHt decrease. On the other hand, the incubation with prazosin was not able to change *m*-synephrine-induced glutathione depletion showing that this effect is independent of α_1-adrenoceptor stimulation. In conclusion, both positional isomers require OCT3 and OCT1-mediated transport to enter into the cardiomyocytes; however, the hydroxyl group in the *p*-position favours the OCT-mediated transport into cardiomyocytes. Furthermore, the structural isomerization of synephrine influences its toxicological profile since only *m*-synephrine caused GSH depletion.

Keywords Synephrine · Phenylephrine · Rat cardiomyocytes · Organic cation transporter · Glutathione depletion

Abbreviations

CORT	Corticosterone
DMSO	Dimethyl sulphoxide
DTNB	5,5-dithio-bis(2-nitrobenzoic acid)
FDA	Food and Drug Administration
GC/IT-MS	Gas chromatography/ion trap-mass spectrometry

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GR	Glutathione reductase
GSH	Reduced glutathione
GSHt	Total glutathione
GSSG	Glutathione disulphide
$HClO_4$	Perchloric acid
HEPES	*N*-(2-hydroxyethyl) piperazine-*N*-(2-ethanesulphonic acid)
IS	Internal standard
LDH	Lactate dehydrogenase
MRP1	Multidrug resistance protein 1
NAC	N-acetyl-cysteine
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
OCT	Organic cation transporter
OCTN	Novel organic cation transporter
PMAT	Plasma membrane monoamine transporter
QUIN	Quinidine
ROS	Reactive oxygen species
SD	Standard deviation
SPE	Solid-phase extraction
TFAA	Trifluoroacetic anhydride
Tiron	4,5-dihydroxy-1,3-benzene disulphonic acid
β-NADH	Reduced β-nicotinamide adenine dinucleotide

Introduction

Synephrine is an unspecific adrenergic agonist, which can exist in three different positional isomers (*ortho o-*, *para p-* and *meta m-*) (Haaz et al. 2006). *o*-Synephrine has not been detected in weight-loss products. Some authors state that, as only *p*-synephrine can be found in nature (Fugh-Berman and Myers 2004; Arbo et al. 2008b; Andrade et al. 2009), *m*-synephrine is not expected to be present in the so-called "natural" dietary supplements. However, the presence of *m*-synephrine in weight-loss products has already been described (Allison et al. 2005; Greenway et al. 2006). The *m*-isoform, also named phenylephrine, is considered the most potent synephrine's adrenergic agonist at α_1-adrenoceptors, when compared to the other positional isomers (Brown et al. 1988).

Synephrine is an adrenergic stimulant, and it is thought to promote weight loss through stimulation of β_3-adrenoceptors, through the increase in fat metabolism rate (Hoffman et al. 2006). The scientific interest towards synephrine increased after the Food and Drug Administration (FDA) prohibition of ephedra-containing weight-loss products. Synephrine emerged as an adequate alternative to ephedrine and soon became one of the most common stimulant present in dietary products (Blanck et al. 2007). However, as with ephedrine, important adverse effects associated with synephrine's consumption in weight-loss products have been reported, mainly at the cardiovascular level. These reports include hypertension, tachyarrhythmia, variant angina, cardiac arrest, QT prolongation, ventricular fibrillation and even acute lateral-wall myocardial infarction (Nasir et al. 2004; Nykamp et al. 2004; Bui et al. 2006; Gange et al. 2006; Thomas et al. 2009). These side effects have already determined the prohibition of synephrine-containing dietary supplements in Canada (Jordan et al. 2004).

Structurally, synephrine is similar to ephedrine, amphetamines, phenylpropanolamine, adrenaline and noradrenaline (Fig. 1). Studies indicate that oxidative stress contributes, directly or indirectly, to the cardiotoxicity of amphetamines and ephedrine (Yamamoto and Zhu 1998; Kovacic and Cooksy 2005). Furthermore, the cardiotoxicity of catecholamines has also been extensively associated with the imbalance of cardiac antioxidant defences (Remião et al. 2001a, b, 2002; Carvalho et al. 2004a, b; Costa et al. 2007, 2009b, c). Therefore, it is possible that the cardiotoxic effects of synephrine can also be related to the decrease in cardiac antioxidants.

Recently, we described the entrance of *m*-synephrine into rat cardiomyocytes (Rossato et al. 2010). However, to the best of our knowledge, no study has been made to determine the mechanism of synephrine transport into the heart or isolated cardiomyocytes. Multispecific organic transporters are responsible for the carrier-mediated transport of a wide range of cationic xenobiotics through cellular membranes. The mediated transport of cations includes several types of transporters, namely the organic cation transporter family (OCT), which includes OCT1, OCT2 and OCT3; and the novel organic cation transporter family (OCTN), which includes the transporters responsible for carnitine uptake OCTN1, OCTN2 and OCTN3 (Wu et al. 2000; Grube et al. 2006; Iwata et al. 2008; McBride et al. 2009). OCT3 is widely distributed, being strongly expressed in the heart. OCT1 is also expressed in human and rodent hearts (Koepsell et al. 2007). When regarding to the OCTN family, only OCTN1 was described in human and rat cardiomyocytes (Wu et al. 2000; Grube et al. 2006; Iwata et al. 2008; McBride et al. 2009). Synephrine is a weak base, and it is well known that organic cations, resulting from being positively charged at physiological pH, are the main substrates translocated by OCTs through the cellular membranes (Koepsell et al. 2007).

In this work, we aimed to investigate the contribution of OCTs in synephrine's entry into rat cardiomyocytes and the effects elicited by the internalized synephrine upon the intracellular glutathione status. We have recently shown that freshly isolated calcium tolerant cardiomyocytes from adult rat are a suitable model for the study of catecholamine transport (Costa et al. 2009a). In addition, the effects

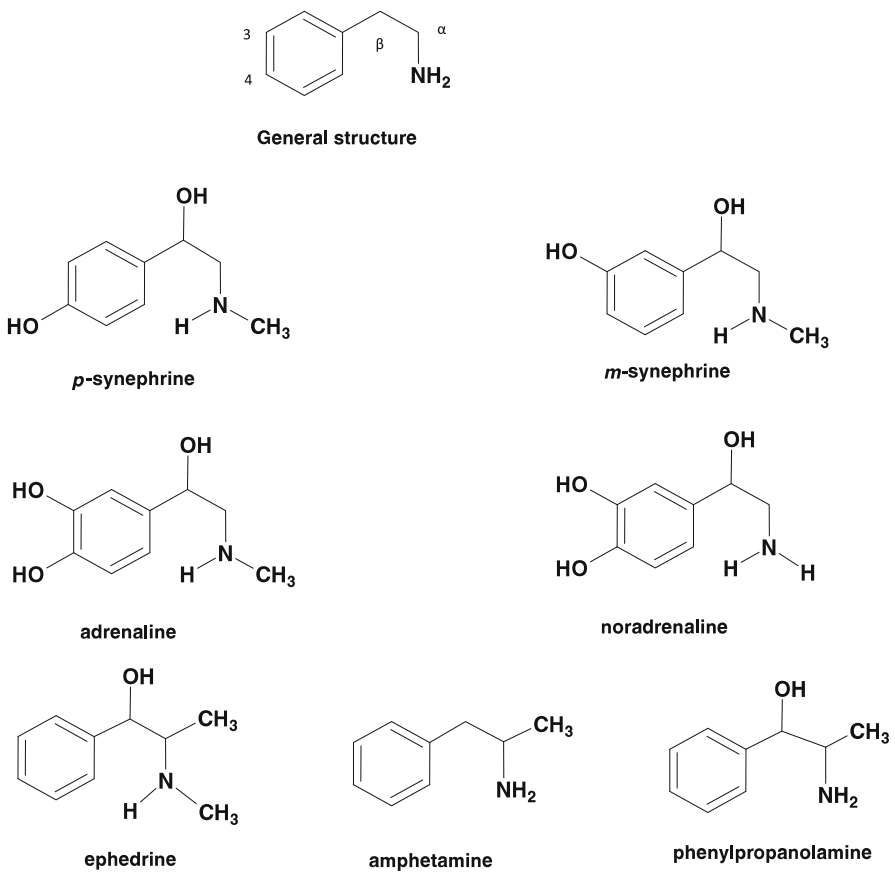

Fig. 1 Chemical structures of compounds with structural similarities with synephrine

upon cell viability, and the levels of total glutathione (GSHt), reduced glutathione (GSH) and glutathione disulphide (GSSG) were determined. Moreover, the possible protective effects of the antioxidants N-acetyl-cysteine (NAC), 4,5-dihydroxy-1,3-benzene disulphonic acid (Tiron) and the contribution of α_1-adrenoceptor activation in synephrine-induced adverse effects were also investigated.

Materials and methods

Animals

Adult male Sprague–Dawley rats (Charles River Laboratories, Barcelona, Spain) weighing 250–350 g were used. The animals were housed in cages, with a temperature- and humidity-controlled environment. Food and water were provided ad libitum, and animals were subjected to a 12-h light–dark cycle. Animal experiments were licensed by Portuguese General Directory of Veterinary Medicine. Housing and experimental treatment of the animals were in accordance with the Guide for the Care and Use of Laboratory Animals from the Institute for Laboratory Research (ILAR 1996). The experiments complied with current Portuguese laws.

Chemicals

All chemicals and reagents were of analytical grade. Trifluoroacetic anhydride (TFAA), (±) *p*-synephrine, (−) *m*-synephrine hydrocloride, 4-hydroxy-3-methoxybenzylamine hydrochloride, collagenase (type IA), *N*-(2-hydroxyethyl) piperazine-*N*-(2-ethanesulphonic acid) (HEPES), quinidine hydrochloride (QUIN), Tiron, NAC, corticosterone (CORT), prazosin, GSH, GSSG, glutathione reductase (GR, EC 1.6.4.2), 2-vinylpyridine, reduced *β*-nicotinamide adenine dinucleotide (*β*-NADH) and 5,5-dithio-bis (2-nitrobenzoic acid) (DTNB) were obtained from Sigma–Aldrich (St. Louis, MO). Collagenase type II was obtained from Worthington (Lakewood, NJ). Dimethyl sulphoxide (DMSO) and perchloric acid ($HClO_4$) were obtained from Merck (Darmstadt, Germany).

Calcium tolerant cardiomyocytes isolation

Calcium-tolerant cardiomyocytes were isolated by Langendorff retro perfusion of adult rat heart, as previously described (Costa et al. 2007, 2009a, b, c). The procedure was based on successive treatments with calcium-free medium and digestion with collagenases (collagenase type II and collagenase type IA in 200 μM calcium-modified Krebs-Henseleit buffer solution), followed by a gentle mechanical disaggregation. Calcium levels were gradually re-introduced until a final concentration of 1 mM in order to obtain calcium-tolerant cardiomyocytes. At the beginning of the experiments, cell viability was always greater than 60%, through evaluation of cardiomyocyte morphology in the optical microscope and by the lactate dehydrogenase (LDH) leakage assay. The obtained viability is in accordance with previous reports for calcium-tolerant cardiomyocytes (Remião et al. 2001a, b; Costa et al. 2007, 2009a, b, c). Incubations were performed in a water bath at 37°C, using a density of 0.25×10^6 viable cells/ml in modified Krebs-Henseleit buffer supplemented with 1 mM $CaCl_2$ (pH 7.4) and saturated with an air stream of carbogen (95% $O_2$5% CO_2), every hour.

After a pre-incubation of 30 min at 37°C, the cardiomyocytes were incubated with the different compounds, namely *p*- and *m*-synephrine (1 mM); the OCT3 inhibitor CORT (100 μM); the OCT1 inhibitor QUIN (100 μM); the α_1-adrenoceptor antagonist prazosin (100 nM); and the antioxidants NAC (1 mM) and Tiron (100 μM).

Cell viability assays

Morphology

The percentage of rod-shaped cells was determined using a Neubauer chamber, as previously described (Remião et al. 2001a, b). Cells with a length/width ratio of >4 were considered rod-shaped cells.

LDH leakage assay

The LDH leakage assay was directly performed to evaluate the level of cell injury at time zero (immediately after addition of the compounds) and after the 3 h incubation in all groups (Remião et al. 2004; Costa et al. 2007).

Sample treatment

After an incubation period of 3 h, cell samples were obtained after centrifugation of cardiomyocyte suspensions, at 18*g*, for 2 min. The pellet was washed 3 times with modified Krebs-Henseleit buffer supplemented with 1 mM $CaCl_2$, centrifuged at 18*g* for 2 min and finally treated according to the different determinations. Washing solutions, obtained after centrifugation, were discarded (Costa et al. 2007, 2009a).

GC/IT-MS analysis

After the 3 h incubation period with 1 mM *p*- or *m*-synephrine, the intracellular content of each synephrine isomer was determined by gas chromatography/ion trap-mass spectrometry (GC/IT-MS), as previously described (Rossato et al. 2010). Cardiomyocyte suspensions were treated as described in the Sect. "Sample treatment", lysed with 5% $HClO_4$ and centrifuged (16,000*g,* 10 min, 4°C). The supernatants obtained were used to evaluate the synephrine's internalization in the cardiomyocytes. Before a solid-phase extraction (SPE) step, all samples were spiked with 10 μl of internal standard (IS) 4-hydroxi-3-methoxybenzylamine hydrochloride and then analysed by GC/IT-MS, as previously described (Rossato et al. 2010).

Quantitative measurements of intracellular *p*- and *m*-synephrine content were carried out by interpolation in the standard curves obtained by injection of standard solutions of both isomers. A new standard curve was prepared each day that the analysis was carried out.

p-Synephrine was dissolved in DMSO. When analysing *p*-synephrine, a group of cells was only incubated with the vehicle (DMSO 1%) in order to guarantee the absence of interfering peaks in the retention times of analytes or IS.

Evaluation of the role of OCT3 and OCT1 in synephrine's transport into the cardiomyocytes

To evaluate the role of OCT3 in the entrance of *p*- and *m*-synephrine into cardiomyocytes, the classical and specific inhibitor CORT was employed. We used 100 μM of CORT since this concentration is described as specific for OCT3 in rat cardiomyocytes (Obst et al. 1996, 2009a). QUIN (100 μM) is described as an OCT1 inhibitor (Costa et al. 2009a) and was used in order to test the influence of this carrier in synephrine-mediated transport into freshly isolated cardiomyocytes.

Cardiomyocytes were exposed to *p*- or *m*-synephrine (1 mM) in the presence or absence of CORT or QUIN. The tested inhibitors were pre-incubated for 30 min before the addition of the synephrine isomers to the cardiomyocyte suspensions.

Samples were analysed by GC/IT-MS after precipitation with 5% $HClO_4$, as described in GC/IT-MS analysis section. Control cells with no treatment and cells exposed to inhibitors alone were analysed in parallel in order to guarantee the absence of interfering peaks in the retention times of analytes or IS.

Evaluation of GSHt, GSH and GSSG levels

The intracellular levels of GSH and GSSG in cardiomyocytes were evaluated by the DTNB-GSSG reductase recycling assay, as previously described (Costa et al. 2007). After a 3 h incubation period, aliquots of cell suspensions were handled as described in Sect. "Sample treatment" above. The obtained cardiomyocytes were lysed and proteins were precipitated with 5% $HClO_4$. After centrifugation (16,000*g*, 10 min, 4°C), the supernatant obtained was used for the determination of GSHt, GSH and GSSG in all groups. For this biochemical determination, the cells were divided into 4 different groups: (1) control cells, with no treatment; (2) cells incubated with synephrine alone; (3) cells pre-incubated for 30 min with the reactive oxygen species (ROS) scavenger (100 μM Tiron or 1 mM NAC) plus synephrine; and (4) ROS scavenger alone (Oosthuizen and Greyling 1999; Carvalho et al. 2004a, b, 2009b, c). These determinations were made for both synephrine isomers, independently. DMSO (1%) was required to prepare *p*-synephrine solutions. Thus, one group of cells was exposed only to DMSO (1%) when *p*-synephrine was tested. Furthermore, to evaluate whether the generation of ROS was related to α_1-adrenoceptors activation, independent experiments were performed only with *m*-synephrine; cardiomyocytes were pre-incubated for 30 min with the α_1-antagonist prazosin (100 nM) (Amin et al. 2001; Costa et al. 2009b, c), followed by the incubation with *m*-synephrine (1 mM).

Protein determination

Cardiomyocyte suspensions were treated as described in the Sect. "Sample treatment", lysed with 5% $HClO_4$ and centrifuged (16,000*g*, 10 min, 4°C). The obtained pellet was dissolved in 0.3 M NaOH, and after homogenization, the protein levels were determined spectrophotometrically, as previously described by Lowry et al. (1951) using a microplate reader (wavelength = 750 nm).

Statistical analysis

Results are presented as means ± standard deviation (SD) from independent experiments with cell suspensions obtained from different rats. Non-parametric tests were used. Statistical comparisons between groups were performed with Kruskal–Wallis test (one-way ANOVA on ranks) followed by the Student–Newman–Keuls *post hoc* test, once a significant *P* had been obtained.

When only two treatment groups were compared, the Mann–Whitney rank sum test was used. Details of the statistical analysis are described in each figure legend. Significance was accepted at *P* values < 0.05.

Results

Incubation with 1 mM of *m*- or *p*-synephrine leads to different intracellular concentrations

As can be seen in Fig. 2, there are significant differences between the intracellular levels of each synephrine isomer. When cardiomyocytes were incubated with 1 mM *m*- or *p*-synephrine for 3 h, the intracellular content of *p*-synephrine was significantly higher (3.3 ± 1.9 μM/mg of protein) when compared with *m*-synephrine levels (1.6 ± 1.1 μM/mg of protein) (Fig. 2).

Inhibition of OCT3 and OCT1 leads to the decrease in the intracellular content of *m*-synephrine

The intracellular content of *m*-synephrine was significantly reduced by the pre-incubation, for 30 min, with the OCT3 inhibitor, CORT (100 μM) (53.7% ± 13.9) and with OCT1 inhibitor, QUIN (100 μM) (54.7% ± 37.0) when compared with the *m*-synephrine group (Fig. 3). These data indicate that both OCTs present in cardiomyocytes isolated from adult rat are involved in the mediated transport of *m*-synephrine.

In order to guarantee the selectivity of CORT at 100 μM, we also performed experiments with 1 and 10 μM of CORT. There were no differences in the magnitude of inhibition of *m*-synephrine's entrance into cardiomyocytes at any of the tested CORT concentrations (data not shown). QUIN, at 100 μM, does not inhibit OCT3 (Costa et al. 2009a).

DMSO (1%) was required to prepare CORT solutions. No interfering peaks in the chromatogram for *m*-synephrine evaluation were found in control groups, CORT, QUIN, or vehicle group (DMSO) (data not shown).

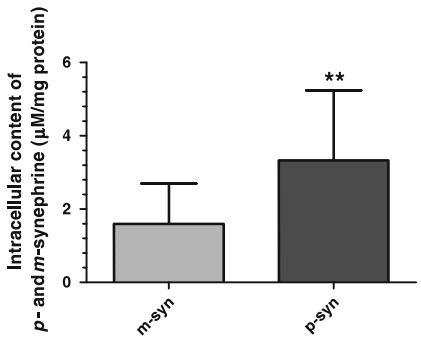

Fig. 2 Comparison of intracellular levels of *m*- and *p*-synephrine in cardiomyocytes incubated for 3 h with 1 mM *m*-synephrine or 1 mM *p*-synephrine. Results are presented as means (μM/mg of protein) ± SD from 16 different experiments. Statistical comparison was made using the Mann–Whitney rank sum test (**$P < 0.01$ vs. *m*-syn)

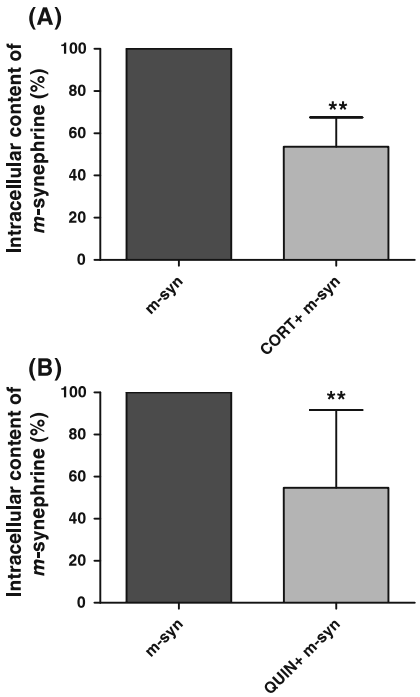

Fig. 3 **a** Intracellular content of *m*-synephrine (*m*-syn) in cardiomyocytes incubated for 3 h with 1 mM *m*-syn and 1 mM *m*-syn + 100 μM CORT. Results are presented as means (%) ± SD from 6 different experiments. **b** Intracellular content of *m*-synephrine (*m*-syn) in cardiomyocytes incubated for 3 h with 1 mM *m*-syn and 1 mM *m*-syn + 100 μM QUIN. Results are presented as means (%) ± SD from 7 different experiments. Statistical comparison was made using the Mann–Whitney rank sum test (**$P < 0.01$ vs. *m*-syn)

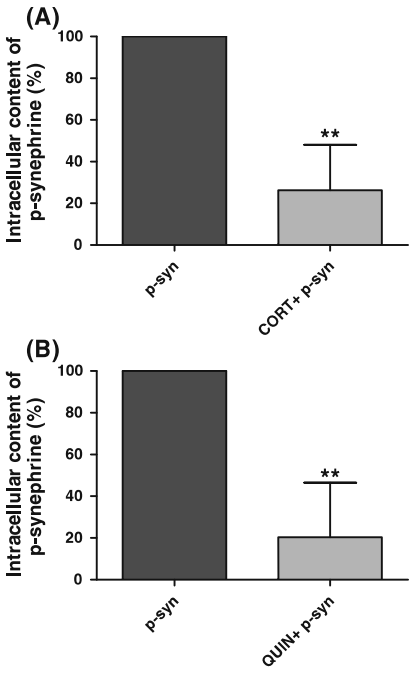

Fig. 4 **a** Intracellular content of *p*-synephrine (*p*-syn) in cardiomyocytes incubated for 3 h with 1 mM *p*-syn and 1 mM *p*-syn + 100 μM CORT. Results are presented as means (%) ± SD from 6 different experiments. **b** Intracellular content of *p*-synephrine (*p*-syn) in cardiomyocytes incubated for 3 h with 1 mM *p*-syn and 1 mM *p*-syn + 100 μM QUIN. Results are presented as means (%) ± SD from 8 different experiments. Statistical comparison was made using the Mann–Whitney rank sum test (**$P < 0.01$ vs. *p*-syn)

Inhibition of OCT3 and OCT1 leads to the decrease in the intracellular content of *p*-synephrine

The intracellular levels of *p*-synephrine were also significantly reduced by the pre-incubation, for 30 min, with CORT (26.3% ± 21.8) and QUIN (20.4% ± 26.2) (Fig. 4). Similar to what happened with the *m*-isoform, both OCT1 and OCT3 have an important role in the mediated transport of *p*-synephrine into cardiomyocytes.

DMSO (1%) was required to prepare CORT and *p*-synephrine solutions. No interfering peaks in the chromatogram for *p*-synephrine evaluation were found in control groups, CORT, QUIN, or vehicle group (DMSO) (data not shown).

Only *m*-synephrine elicits alteration in the glutathione status

In Fig. 5, the levels of GSHt in cardiomyocytes after a 3 h incubation period in control (13.7 ± 2.1 μM/0.25 × 10^6 cardiomyocytes), vehicle (DMSO) (12.2 ± 2.5 μM/0.25 × 10^6 cardiomyocytes) and 1 mM *p*-synephrine groups (12.4 ± 1.4 μM/0.25 × 10^6 cardiomyocytes) can be observed. There are no statistical differences between groups.

When cells were incubated with 1 mM of *m*-synephrine, a significant difference can be observed in GSHt and GSH levels when compared with control group, as shown in

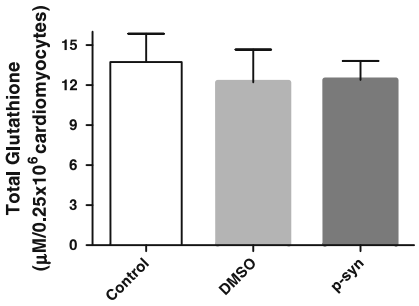

Fig. 5 Total glutathione (GSHt) intracellular levels in cardiomyocytes in control group and in cardiomyocytes incubated with 1 mM *p*-synephrine (*p*-syn) for 3 h. DMSO was used as vehicle. Results are presented as means ± SD from 3 different experiments. No statistical differences were observed between groups

Fig. 6. The GSHt and GSH depletion induced by *m*-synephrine in cardiomyocytes was counteracted by the presence of the antioxidants Tiron (100 μM) (Fig. 6a, b) and NAC (1 mM) (Fig. 6c, d). Cardiomyocytes incubation with Tiron, a superoxide scavenger, led to the intracellular increase in GSH at 3 h in the Tiron+ *m*-synephrine group (8.40 ± 3.12 μM GSH/0.25 × 10^6 cardiomyocytes) when compared with only *m*-synephrine-treated cells (5.8 ± 2.4 μM GSH/0.25 × 10^6 cardiomyocytes) (Fig. 6b). The GSH values in the Tiron+ *m*-synephrine group were similar to those found in the control group at 3 h (10.8 ± 2.9 μM GSH/0.25 × 10^6 cardiomyocytes) (Fig. 6b). The same tendency occurred in the GSHt values (Fig. 6a). These results show that the pre-incubation with Tiron was able to prevent the alterations in glutathione status observed in *m*-synephrine groups. Regarding NAC + *m*-synephrine group, the intracellular levels of GSH were significantly higher in this group (7.6 ± 1.6 μM GSH/0.25 × 10^6 cardiomyocytes) when compared to *m*-synephrine group (5.1 ± 1.9 μM GSH/0.25 × 10^6 cardiomyocytes) (Fig. 6d). In fact, the GSH values of NAC + *m*-synephrine group were similar to those of the control group after 3 h incubation (8.52 ± 1.3 μM GSH/0.25 × 10^6 cardiomyocytes). The GSHt levels between groups show the same tendency. No significant differences were observed in the intracellular levels of GSHt and GSH in cardiomyocytes incubated with Tiron and NAC alone when compared with the control groups (Fig. 6).

Moreover, the GSHt and GSH decrease promoted by *m*-synephrine was shown to be independent from α_1-adrenoceptors stimulation, since pre-incubation with prazosin (100 nM) did not prevent or counteracted the decreases observed in GSHt and GSH intracellular levels of *m*-synephrine group ($N = 3$) (data not shown).

No significant changes were observed in the GSSG intracellular levels in any of the tested groups (data not shown).

Cellular viability

During the 3 h incubation period, none of the treatments caused changes in cell viability when compared to control, as verified by the LDH leakage assay (data not shown).

Discussion

The major findings of this work highlighted the role of OCT3- and OCT1-mediated transport of synephrine into freshly isolated cardiomyocytes from adult rat and showed that the different positional isomers of synephrine have different toxicological profiles, since only the *m*-isoform elicited significant changes in the intracellular glutathione levels.

In our previous study, it was shown, for the first time, the ability of *m*-synephrine to be transported into cardiomyocytes (Rossato et al. 2010). In the present work, we not only confirmed the *m*-synephrine internalization into the cardiomyocytes but also of the *p*-synephrine's (Fig. 2). Thus, we tried to elucidate the mechanisms involved in the transport of both synephrine's positional isomers used for weight-loss purposes. The results obtained showed that *m*-synephrine is transported by OCT3 and OCT1 into cardiomyocytes, as highlighted by lower *m*-synephrine intracellular levels detected when cells were pre-incubated, for 30 min, with OCT3 and OCT1 inhibitors, CORT and QUIN, respectively (Fig. 3). Furthermore, no differences were observed in the amount of inhibition obtained with CORT or QUIN; hence, OCT3 and OCT1 have similar importance in the entrance of this particular isomer. In the same way, *p*-synephrine is also transported into cardiomyocytes by OCT3 and OCT1 since similar lower *p*-synephrine intracellular levels were detected in the presence of CORT and QUIN (Fig. 4).

The substrate and inhibitor specificities of OCTs and OCTNs overlap extensively (Koepsell et al. 2007). Although CORT shows dissimilar affinity and selectivity to the transporters of different animal species (Hayger-Zillgen et al. 2002), CORT, at the employed concentration (100 μM), is widely described in the literature as a specific and selective inhibitor of OCT3 in rat-isolated cardiomyocytes (Obst et al. 1996; Costa et al. 2009a). QUIN could be considered an unspecific inhibitor, once it has already been described as an inhibitor of OCT1 (Koepsell 2004), OCTN2 (Ohashi et al. 1999), and of the plasma membrane monoamine transporter (PMAT) (Engel and Wang 2005). The OCTN family is responsible for carnitine uptake. Heart muscle is highly dependent on active carnitine uptake from the blood (Grube et al. 2006). However, only OCTN1 is described in rat and human cardiomyocytes (Wu et al. 2000; Grube et al. 2006; Iwata et al. 2008; McBride et al. 2009). PMAT is also able to transport organic cations, and despite its mRNA description in the heart (Engel et al. 2004; Engel and Wang 2005), to the best of our knowledge, the presence of its protein in cardiomyocytes has never been reported. Moreover, we already demonstrated that QUIN (100 μM) does not inhibit OCT3 in calcium-tolerant cardiomyocytes freshly isolated from adult rat (Costa et al. 2009a). Thus, we can assume that QUIN (100 μM) inhibits only OCT1 transport in this cellular model.

The involvement of OCT3 in the uptake of adrenaline is well known (Costa et al. 2009a), and taking into account the structural similarities between the chemical structures of synephrine and adrenaline (Fig. 1), it is possible to

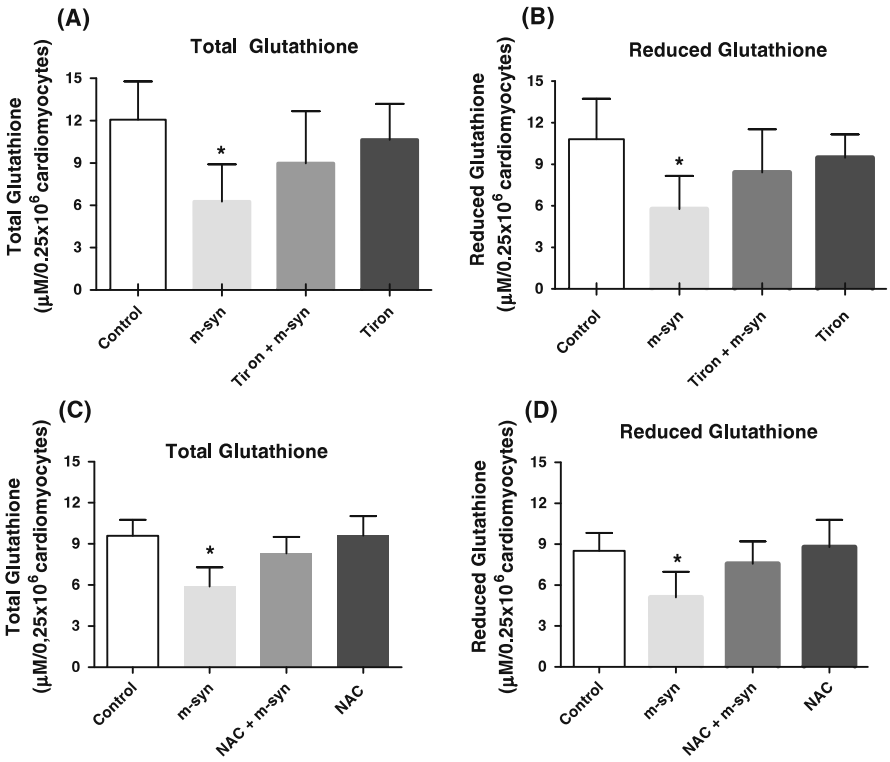

Fig. 6 **a** Total glutathione (GSHt) in cardiomyocytes in control, 1 mM *m*-synephrine (*m*-syn), 1 mM *m*-synephrine with 100 µM Tiron (Tiron+ *m*-syn) and 100 µM Tiron groups after a 3 h incubation. **b** GSH intracellular levels in cardiomyocytes in control, 1 mM *m*-synephrine (*m*-syn), 1 mM *m*-synephrine with 100 µM Tiron (Tiron+ *m*-syn) and 100 µM Tiron groups after a 3 h incubation. **c** GSHt intracellular levels in cardiomyocytes in control, 1 mM *m*-synephrine (*m*-syn), 1 mM *m*-synephrine with 1 mM NAC (NAC + *m*-syn) and 1 mM NAC groups after a 3 h incubation. **d** GSH intracellular levels in cardiomyocytes in control, 1 mM *m*-synephrine (*m*-syn), 1 mM *m*-synephrine with 1 mM NAC (NAC + *m*-syn) and 1 mM NAC groups after a 3 h incubation. Results are presented as means (µM) ± SD from 5 different experiments. Statistical comparisons were made using Kruskal–Wallis test, followed by the Student–Newman–Keuls *post hoc* test (*$P < 0.05$ vs. control)

postulate that the side chain is of outmost relevance for the OCT3-mediated transport into the cardiomyocytes. On the other hand, OCT1 is not related to the transport of adrenaline in cardiomyocytes (Costa et al. 2009a), suggesting that the presence of a single hydroxyl group in the aromatic ring probably favours the OCT1-mediated transport. Interestingly, starting from the same initial concentration of both positional isomers (1 mM), the intracellular levels detected in cardiomyocytes after 3 h incubation were significantly higher for *p*-synephrine when compared with *m*-synephrine (Fig. 2). This result suggests that OCT3 and OCT1 share similarities for their substrates, but the hydroxyl group in the *p*-position favours the OCT-mediated transport into cardiomyocytes.

Although the incubation with *p*-synephrine results in significantly higher intracellular level of this isomer in the cardiomyocytes than those obtained with the same concentration of the *m*-isoform (Fig. 2), *p*-synephrine did not promote any significant changes in the intracellular glutathione levels (Fig. 5). This highlights that the structural isomerization influences the toxicological profile of synephrine. This result can in fact corroborate the low or negligible cardiotoxicity of *p*-synephrine (Arbo et al. 2008a, b, 2009). Even using the same elevated concentration of *p*-synephrine (1 mM), which is justified by our short-therm study, lower intracellular levels of synephrine were found in *m*-synephrine group. Moreover, this low *m*-synephrine intracellular concentration elicited significant GSHt and GSH depletion (Fig. 6). Glutathione depletion has been associated with electrophysiological remodelling in cardiomyocytes from rat and in canine in vivo models (Sridhar et al. 2009; Zheng et al. 2010). This is an important remark since this imbalance in cardiac glutathione levels can increase the risk of arrhythmias, myocardium infarction and cardiac death (Sridhar et al. 2009; Zheng et al. 2010).

Furthermore, we were able to totally avoid *m*-synephrine-induced imbalance in intracellular glutathione levels

with two different antioxidants, NAC (1 mM) and Tiron (100 μM) (Fig. 6). NAC is a powerful nucleophile that directly scavenges several ROS (Halliwell and Gutteridge 1998) and is also the acetylated precursor of GSH. It can replenish glutathione in studies with longer incubation periods (Forman et al. 1988); however, in the present study, this effect upon glutathione synthesis is not relevant considering the short incubation period of 3 h. Tiron is a more specific scavenger, which acts on the superoxide anion, and is considered a superoxide dismutase mimetic (Ghosh et al. 2002). Altogether, our results demonstrate that the glutathione depletion observed when cardiomyocytes were incubated with *m*-synephrine (1 mM) for 3 h was related to increases in intracellular ROS formation. In order to investigate whether the glutathione depletion promoted by *m*-synephrine was related to the α_1-adrenoceptors activation, we performed a study with prazosin at a concentration (100 nM) previously described to selectively antagonize α_1-adrenoceptors (Amin et al. 2001; Costa et al. 2009b). As already mentioned, *m*-synephrine is a potent α_1-agonist (Brown et al. 1988), and it is well known that the stimulation of α_1-adrenoceptors leads to increases in intracellular ROS through the reduced β-nicotinamide adenine dinucleotide phosphate (NADPH) oxidase pathway (Amin et al. 2001). We showed that the effects of *m*-synephrine on glutathione levels were independent of α_1-adrenoceptors stimulation, since co-incubation with prazosin did not prevent the changes in the glutathione status induced by the isomer. The increases in intracellular ROS could be related to the auto-oxidation of *m*-synephrine. In fact, the oxidation of synephrine through the tyrosinase pathway was already described in a study with mushroom tyrosinase (Garcia-Carmona et al. 1987).

Interestingly, GSH depletion induced by *m*-synephrine was not accompanied by a corresponding increase in GSSG levels. One possibility that could explain this finding is that the GSSG formed can be exported to the extracellular medium. It was reported that the multidrug resistance protein 1 (MRP1) transporter, present in cardiomyocytes, might have a role in the cellular response to oxidative stress, namely mediating the cellular efflux of GSH conjugates and GSSG (Cole and Deeley 2006; Costa et al. 2009a). Another hypothesis is the formation of synephrine's GSH-conjugates, similar to what is observed with adrenaline (Costa et al. 2007), that decrease intracellular GSH with no changes in GSSG levels.

Although toxicological studies concerning synephrine are still scarce, existing case reports suggest a cardiotoxic potential for it (Jordan et al. 2004; Nasir et al. 2004; Nykamp et al. 2004; Bui et al. 2006; Gange et al. 2006; Thomas et al. 2009). The toxicity of *m*-synephrine is better understood considering its use as a decongestant (Martindale 2004; Santana et al. 2008), a vasopressor agent and a mydriatic agent (Haaz et al. 2006). Frequently, the cardiac effects of *m*-synephrine are related to the pronounced stimulation of α_1-adrenoceptors (Thomas and Tripathi 1986; Terzic and Vogel 1991; Rang et al. 2007; Navarro-Sobrino et al. 2010). However, in the present study, we observed that the cardiac adverse effects induced by the *m*-isoform of synephrine in isolated cardiomyocytes are independent of α_1-adrenoceptors stimulation. Despite the more abundant entrance of *p*-synephrine into cardiomyocytes, this positional isomer seems to be innocuous under the experimental conditions. In vivo studies also demonstrated a negligible toxicity of *p*-synephrine (Arbo et al. 2008a, b, 2009). In fact, our results corroborate that *m*- and *p*-synephrine clearly show different toxicological profiles. Although *m*-synephrine can also be found in weight-loss products (Allison et al. 2005; Greenway et al. 2006), most safety studies concerning synephrine's use in weight-loss only evaluate the *p*-isoform (Arbo et al. 2008a, b, 2009). This fact could explain the discrepancy between existing toxicological studies, which indicates the low toxicity of *p*-synephrine, and the important case reports of cardiotoxicity concerning synephrine-containing weight-loss products.

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