Adenosine modulates nitric oxide levels though activation of adenosine A_1 receptor in cell culture dorsomedial medulla oblongata

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Abstract

Adenosine and nitric oxide act regulating the fine tuning of cardiovascular control in the nucleus tractus solitarii (NTS). Although the relationship between NO and adenosine in the peripheral system is well established in the literature, this relationship within the NTS is little explored. In the present study, we show the interaction between the adenosinergic and nitrergic systems in dorsomedial medulla oblongata cell culture of Wistar Kyoto (WKY) and Spontaneously Hypertensive Rats (SHR) through molecular and cellular technical. We observed that administration of adenosine A_1 receptor (A_1R) agonist, CPA, induced a decrease of nitrite and nNOS mRNA levels in dorsomedial medulla oblongata cell culture of WKY and SHR rats. This decrease in nitrite levels was attenuated by the pretreatment with the A_1R selective antagonist, CPT. Our experiment showed that the cAMP-PKA pathway is involving in A_1R mediates nitrite levels decreasing in SHR and WKY cells. Finally, downregulation A_1R revealed that the reduction in A_1R expression increased nNOS mRNA levels compared with non-silence control in cells from WKY and SHR. In summary, our results highlights the importance of the modulatory role of the adenosine A_1R subtype on nitric oxide in cultured dorsal medulla oblongata cells of WKY and SHR rats.

1. Introduction

Adenosine and nitric oxide (NO) acts as important neuromodulators at many levels of the central nervous system, including the nucleus tractus solitarius (NTS). The NTS is one of the main nuclei responsible for integrating different signals from other brains areas or organs in order to originate a specific and orchestrated autonomic response. In this nucleus, adenosine and NO decreases blood pressure (BP), heart rate (HR), and renal sympathetic nerve activity (Tseng et al. 1996; Mosqueda-Garcia et al. 1991). The relationship between adenosine A_{2a} receptor and NO is well documented in the literature, mainly in the NTS. However, the lack of correlation between adenosine A_1R subtype stimulation and NO release in NTS deserves attention.

Stimulation of adenosine A_1R within the NTS evokes pressor responses and increases the pulse pressure since these responses were selectively bloked by DPCPX, a selective A_1R antagonist (Barraco and Phillis, 1991). Studies by Scislo and O'Leary (2002) also reported that activation of adenosine A_1R evokes predominantly pressor and sympathoexcitatory responses in NTS. On the other hand, microinjection of S-nitrosocysteine, a NO donor, in the NTS of awake rats, produced a decrease in arterial pressure and heart rate (Machado and Bonagamba, 1992a). Similarly, unilateral microinjection of L-arginine into the NTS produced prominent dose-related depressor and bradycardic effects and reduced renal sympathetic nerve activity (Tseng et al. 1996). These cardiovascular effects produced by L-arginine are inhibited by pharmacological interventions that block NO production (Tseng et al. 1996).

Studies from our laboratory have reported that adenosine A_1R are heterogeneously distributed within the NTS in adults (Carrettiero and Fior-Chadi, 2004) and young rats (Carrettiero and Fior-Chadi, 2008) and it is suggested that this might contribute to the development of hypertension in the SHR. Indeed, recent work in our laboratory showed that activation of adenosine $A_{2a}R$ subtype increases nitrite levels and nNOS mRNA expression in cultured cells from dorsal medulla oblongata of WKY and SHR (Costa et al. 2013).

The aim of the present study was to analyze the modulation of the nitrergic system by adenosine A_1R activation. Therefore, we have evaluated nitrite production and nNOS mRNA following treatment with CPA, as well as the analysis of the intracellular pathway involved in this modulation in cultured cells from dorsal medulla

oblongata of WKY and SHR rats. Our study reports that the A_1R activation modulates the nitrite production and nNOS mRNA expression.

2. Results

Effects of stimulation of adenosine A_1R on nitrite production

The effects of the A_1R agonist (CPA) on nitrite levels in cultured cells of the dorsomedial medulla oblongata of WKY and SHR rats are presented in figure 1. The A_1R activation using the agonist CPA induced a significant decrease in nitrite levels in all concentrations and time evaluated when compared to their respective controls. At 6 h, WKY cells showed an abrupt reduction in nitrite levels at 0.1 µM while in SHR cells this concentration of CPA did not affect the nitrite levels. On the other hand, the responses to treatment with CPA at 1 µM and 10 µM were similar in WKY and SHR cells. At 12 h of treatment, the nitrite levels were significantly reduced at all concentrations tested in WKY and SHR cells when compared to control. At 24 h of treatment, both strains showed pronounced reduction in nitrite levels at 0.1 µM compared to their respective controls. After this reduction, nitrite levels were kept constant at 1 µM and 10 µM in WKY and SHR cells. When cells were pretreated the A_1R antagonist, CPT, the effect promoted by CPA was completely abolished. CPT treatment alone did not change nitrite levels when compared to control (Fig 2).

Effects of inhibition of intracellular signaling molecules on nitrite levels

To evaluate which intracellular pathway is involved with decreased nitrite levels in medulla oblongata cultured cells evoked by A_1R stimulation, pharmacological treatments were performed using different inhibitors and activators of intracellular signaling molecules in the presence and absence of CPA, at the concentration of 0.1 µM for 12 h. The response pattern to A_1R stimulation was similar in WKY and SHR (Fig 2). SQ22536, an AC inhibitor, exacerbated the effect of CPA on nitrite production compared with CPA alone. On the other hand, 6Bnz, a PKA activator, reversed the effect induced by CPA on nitrite levels. SQ22536 and 6Bnz alone did not induce any changes in nitrite concentration (not showed).

Effect of stimulation of adenosine A_1R on nNOS mRNA expression

The expression of nNOS mRNA was also analyzed to evaluate modulation of the enzyme after stimulation of A_1R by CPA. In both strains, nNOS mRNA expression levels exhibited a decrease in response to CPA treatment after 6, 12 and 24 h of exposure only at 10 µM concentration (Fig. 3) when compared to respective control (Fig. 3B and 3C). It can be observed that nNOS expression is already very much decreased in the SHR in comparison to the WKY rat.

Effect of knockdown of the adenosine A_1R on nNOS mRNA expression

Since we observed a change in nNOS mRNA expression levels after stimulation of A_1R in cell culture from the dorsal brainstem of neonates WKY and SHR, we decided to evaluate the effects of A_1R knockdown on the nNOS mRNA expression. Lentiviral vectors were added to the cultures for 5 h. GFP-expressing cells were observed 4 days after infection with the neurons in good condition at multiplicity of infection (m.o.i.) of 15 (Fig. 4). A_1R shRNA produced a significant knockdown of A_1R expression in cells from both strains, 39,2% in WKY and 24,5% in SHR, when compared with non-silence control (Fig. 4A). The A_1R knockdown was followed by an increase in nNOS mRNA levels, 29,5% in WKY and 59% in SHR, compared with non-silence control (Fig. 4B).

3. Discussion

The current studies give a new insight with respect to modulatory role of the A_1R on NO release taking into account nitrite production, intracellular signaling and involvement of nNOS in cultured dorsomedial medulla oblongata cells of neonate WKY and SHR rats. The most important finding in the present study were: (1) the activation of the adenosine A_1R decrease the nitrite production, (2) the cAMP-PKA pathway is involved in the nitrite decrease mediated by A_1R activation, (3) the activation of the adenosine A_1R modulate nNOS mRNA expression and (4) knockdown.

We showed that administration of CPA, A_1R agonist, induces a dose-dependent decrease in nitrite levels in WKY and SHR cells and this reduction elicited by the A_1R activation was completely and selectively blocked by the selective A_1R antagonist,

CPT. Higher concentrations of CPA did not increase further the inhibitory action of CPA. It is suggested that A_1R-mediated reduction in NO levels must be involved in pressor and sympathoexcitatory responses by A_1R stimulation in NTS, since an intact synthesis of NO is necessary to develop depressor and sympathoinhibitory responses. Possibly the reduction of nitric oxide synthesis in NTS might be the mechanism involved in the pressor component of the response mediated via A_1R activation.

We evaluated if A_1R stimulation-induced decrease in nitrite levels in cultured cells from medulla oblongata was dependent of PKA pathway. Our data suggest that A_1R exerts inhibitory effect in nitrite levels through activation of the classical pathway of the PKA, since inhibition of adenylyl cyclase by SQ22536 exacerbated the nitrite decrease and PKA activator, 6Bnz, attenuated the ability of CPA to reduce the nitrite levels. It's known that A_1R belongs to a family of G-protein coupled receptors (GPCR), specifically G_i and that A_1R activation leads predominantly to inhibition of adenylyl cyclase (Burnstok, 2007). Thus, the observed inhibitory effects of CPA on nitrite accumulation might be due to the inhibition of adenylyl cyclase via activation of the A_1 receptor.

Here, we showed a decreased basal nNOS mRNA expression in SHR cultured cells in comparison to the control WKY, in contrast to that reported by Ferrari and Fior-Chadi (2005) and Edwards et al. (2004). However, this contradiction might be due to the different age of rats, since we have used neonate rats and the previous studies used young pre-hypertensive rats or even old animals with established hypertension. Our study also showed that activation of A_1R by CPA induced a decrease in nNOS mRNA levels in higher concentration of the agonist, at 10 µM in both strains. It is possible that there is a dynamic between A_1R and $A_{2a}R$ activation, since that previous study by our laboratory (Costa et al. 2013) showed lower concentration of the $A_{2a}R$ agonist, CGS21680 induced an increase in nNOS mRNA levels. It is know the involvement of the nNOS isoform in the depressor and sympathoinhibitory responses. Carvalho et al. 2006 have studied nNOS knockout mice and reported that there was a significant reduction of baroreflex responses when compared with wild-type mice. Indeed, Lin et al. 2012 using shRNA for nNOS showed that loss of nNOS expression in NTS is associated with a decreases baroreflex response. Our observation that an early difference in nNOS expression in SHR cells together with an imbalance between A_1R and $A_{2a}R$ activation might collaborate with the development of high blood pressure in these animals.

Although A_1R agonists and antagonists have provided important insights into the various functions of this receptor, including those shown in this study, the uncertainty of their selectivity can generate questions about their effects. Thus, to elucidate the function of many receptors with greater specificity, new genetic tools have emerged, such as the RNA of interference. When we knocked down the expression of A_1R in SHR and WKY cultured cells using RNA of interference, we observed a decrease in the A_1R mRNA levels in both strains and an increase in nNOS mRNA levels after knockdown of A_1R. It is possible that down-regulation of A_1R has resulted in up-regulation of $A_{2a}R$. It could be in part responsible for the increased nNOS mRNA levels since that $A_{2a}R$ activation is involved in the increase in nNOS mRNA expression (Costa et al. 2013). Indeed, as A_1Rs are negatively coupled to adenylyl cyclase by inhibitory G proteins, lack of activation of the A_1R might increase nNOS mRNA expression.

Studies by Lin and Talman (2001) reported that glutamate receptors (GluRs) of the AMPA subtype participate in cardiovascular regulation in the NTS and that response to AMPA receptor activation might be linked to NO. Indeed, GluRs and nNOS are colocalized in neurons of NTS and this anatomical relationship might contribute to cardiovascular regulation. Since that pressor and presumably sympathoexcitatory responses has been associated to A_1R-mediated inhibition of glutamate release in the NTS (Scislo and O'Leary, 2002), we speculate if this response might be due to nitric oxide decreased levels after A_1R stimulation. Further studies will be required to explore this possibility.

In conclusion, the present work gives a valuable contribution for understanding the relationship between adenosine A_1R and nitric oxide in cardiovascular control. More important is that our study reports a modulatory effect of A_1R activation on nitrite production and nNOS mRNA expression and this modulation might be performed through PKA pathway.

4. Methods

Animals

Adult male and female WKY rats and SHR from the Department of Physiology, Institute of Biosciences, University of São Paulo, Brazil, were kept in cages (in proportion 1 male to 2 female) under a regular light-dark cycle (light on at 7:00 a.m. and off at 7:00 p.m.) in temperature and humidity-controlled rooms and received food and

water ad libitum. For dorsomedial medulla oblongata cell culture, 1-day-old SHR and WKY neonates were used in the present study (± 12 neonates for experiment). All the procedures and protocols were performed in accordance with Institutional and International Guidelines for Animal Experimentation (protocol number 065/2008).

Dissociated dorsomedial medulla oblongata cell culture

Cell culture methodology was described in detail elsewhere (Kivell *et al*. 2001). Briefly, dorsomedial medulla oblongata portion were dissected out and dissociated in cold isotonic salt solution (NaCl 120 mM; KCl 5 mM; KH_2PO_4 1.2 mM; $MgSO_4$.7H2O 1.2 mM; $NaHCO_3$ 25 mM and glucose 13 mM), pH 7.4. Cells were suspended in Neurobasal A media (Invitrogen) supplemented with L-glutamine (250 mol/L, Sigma), GlutaMax (250 mol/L, Gibco), B27 (2%, Gibco), and gentamicin (40 mg/L, Gibco). Viable cells were counted and plated on poly-D-lysine-coated culture dishes (35 mm, Nunclon, USA) at the concentration of 1800 cells/mm^2. The experiment was repeated three times for each strain using different pools of animal's tissue. Cultures were kept in a humidified incubator with 5% CO_2 and 95% air, at 37°C, for 7 days prior to experimentation. After treatment, the supernatants were used to measure the NO production.

Drugs and treatment

The effect of different concentration of CPA (10^{-5}, 10^{-6}, 10^{-7} M – Sigma, USA) on NO production and nNOS mRNA expression was evaluated in different time (6, 12 and 24 hours) in the present study. The intracellular cascade involved in the effect of CPA on nitrite production was evaluated using different inhibitors and activators of intracellular signaling molecules: SQ22536 (10^{-7} M), an adenylyl cyclase inhibitor – Sigma, USA; 6Bnz (10^{-5} M), a PKA activator – Sigma, USA. Drugs were added 10 min before CPA for evaluation of NO production. Stock solutions were prepared in dimethylsulphode (DMSO) or phosphate buffer sodium (PBS) and diluted in fresh culture medium immediately before use. Concentration of DMSO in the medium did not induce any changes on nitrite production.

Measurement of the NO production

The NO production was evaluated indirectly by accumulation of nitrite in culture supernatants using the DAN (diaminonaphthalene) reaction (Misko et al., 1993).

Briefly, 100 ul of culture medium were incubated with 10 ul of freshly prepared DAN (0.05mg/ml in 0.62M HCl). After 10 min incubation, the reaction was terminated with 10ul of 2.8M NaOH. Formation of the 2,3-diaminonaphthotriazole was measured using fluorescent plate reader with excitation at 365nm and emission read at 450nm. The concentration of nitrite ions was quantified using $NaNO_2$ as standard.

Real-time PCR

Cells had total RNA extracted and purified according to illustra RNAspin protocol (Invitek, Berlim, Germany). Two-step reverse transcriptase PCR was carried out to determine the effects of A2aR agonist treatment on nNOS mRNA. High-quality total RNA was used in reverse transcription reactions to convert RNA to double-stranded cDNA using Reverse Transcription Reaction Kit (Applied Biosystems, USA). Briefly, to 1 ug of total RNA was added TaqMan buffer (1X), $MgCl_2$ (5.5 uM), dNTPs (500 uM), random hexamers (2.5 uM), RNase inhibitor (0.4 U/uL), and the MultiScribe Reverse Transcriptase (1.25 U/uL) to a final volume of 50 uL. Two control tubes were inserted in the assay: one without the template and another without reverse transcriptase. RNA was converted into cDNA after incubations of 10 min at 25°C followed by 30 min at 48°C and 5 min at 95°C in a thermocycler. cDNA was kept at -80°C until its use for real-time PCR. The primers, probe, and reagents for real-time PCR were commercially available at Applied Biosystems (Taqman) (Foster City, CA, USA). Evaluated nNOS mRNA was NOS1 (Taqman assay: RN00583793_m1). Protocol provided by the manufacturer was rigorously followed.

RNA interference

For the RNA interference procedure, cultured cells of the dorsomedial medulla oblongata of WKY and SHR rats were infected by pGIPZ lentiviral vector containing shRNAmir to A_1R (VGH5518-10112715) and non-silencing RNAi as control (RHS4348), purchased from Open Biosystems. Briefly, after three days in culture, cells of the medulla oblongata of WKY and SHR were exposed at approximately $1x10^8$ Transducing Units per ml (TU/ml) for five hours. Three days after the infections, the RNA was extracted and immediately frozen for real time PCR analysis both A_1R and nNOS mRNA levels.

Statistical analysis

Results were analyzed by two-way ANOVA followed by the Bonferroni post hoc test or unpaired Students t test accessed through GraphPad Prism (GraphPad Software Inc., version 5.00, CA, USA). A $p<0.05$ was considered to indicate statistically significant differences. Data are expressed as mean ± standard error of the mean (SEM). All experiments were performed 3 times independently (n=3).

5. Acknowledgement

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7. Legends

Figure 1: Effect of CPA treatment on nitrite production in cultured cells from dorsomedial medulla oblongata of WKY and SHR neonates. (A), concentration-response curve on nitrite production after 6 h of CPA treatment. (B), concentration-response curve after 12 h of CPA treatment. (C), concentration-response curve on nitrite production after 24 h of CPA treatment. At 12 h and 24 h, WKY and SHR cells were responsive at all concentrations. Nitrite determination was performed in the supernatant using the DAN assay. Data are expressed as mean ± SEM (n=3), #$p<0.05$ when compared to WKY basal situation and *$p<0.05$ when compared to SHR basal situation. Two-way ANOVA following Bonferroni post-hoc test was employed in the statistical analysis.

Figure 2: A_1R-mediated nitrite decreases in cultured cells from dorsomedial medulla oblongata are associated with PKA activation. Nitrite levels in response to A_1R agonist and antagonist treatments, as well as AC inhibitor and PKA activator in cultured cells from WKY and SHR after 6 h. The A_1R antagonist, CPT, was added 10 min before the agonist CPA, and was able to abolish CPA-mediated decrease in nitrite levels. SQ22536, AC inhibitor, exacerbated the effect of A_1R agonist, while 6Bnz, PKA activator, reversed CPA-induced nitrite decrease. Data are shown as mean ± SEM (n=3). #$p<0.05$ compared with control from the same strain, *$p<0.05$ compared with CPA treatment. Two-way ANOVA followed by Bonferroni post hoc test was employed in the statistical analysis.

Figure 3: Concentration–response curve illustrate the modulation by CPA of the nNOS mRNA expression levels in cultured cells from dorsomedial medulla oblongata of WKY and SHR rats. (A) CPA exposure for 6 h induced a decrease of nNOS mRNA expression at 10 µM concentration in both strains. CPA treatment for 12 h (B) and 24 h (C) showed the same profile exhibit at 6 h in WKY and SHR. The data

are represents by mean ± SEM (n=3), †p<0.05 when compared with control WKY, #p<0.05 when compared to WKY basal situation and *p<0.05 when compared to SHR basal situation. Two-way ANOVA following Bonferroni's post-hoc test was employed in the statistical analysis.

Figure 4: A_1R knockdown increase nNOS mRNA expression levels in cultured cells from dorsomedial medulla oblongata. The A_1R knockdown was evaluated by analyzing A_1R mRNA levels after infection with pGIPZ lentiviral vector containing shRNAmir to A_1R. In panels above, transduction of neurons with the GFP expressing lentivirus vector. Images were taken 5 days after cell primary culture from the dorsomedial medulla oblongata (m.o.i. = 15). In both strains, A_1R expression levels were reduced compared with non-silence control (4A). On the other land, nNOS mRNA levels were increased in cultured cells infected with shRNAmir to A_1R compared with non-silence culture (4B). Data are represented by mean ± SEM (n=3), #p<0.05 when compared with control WKY, *p<0.05 when compared to SHR non-silence control. Student's t test was employed in the statistical analysis. Scale bar represents 50 µm.

Figure 1

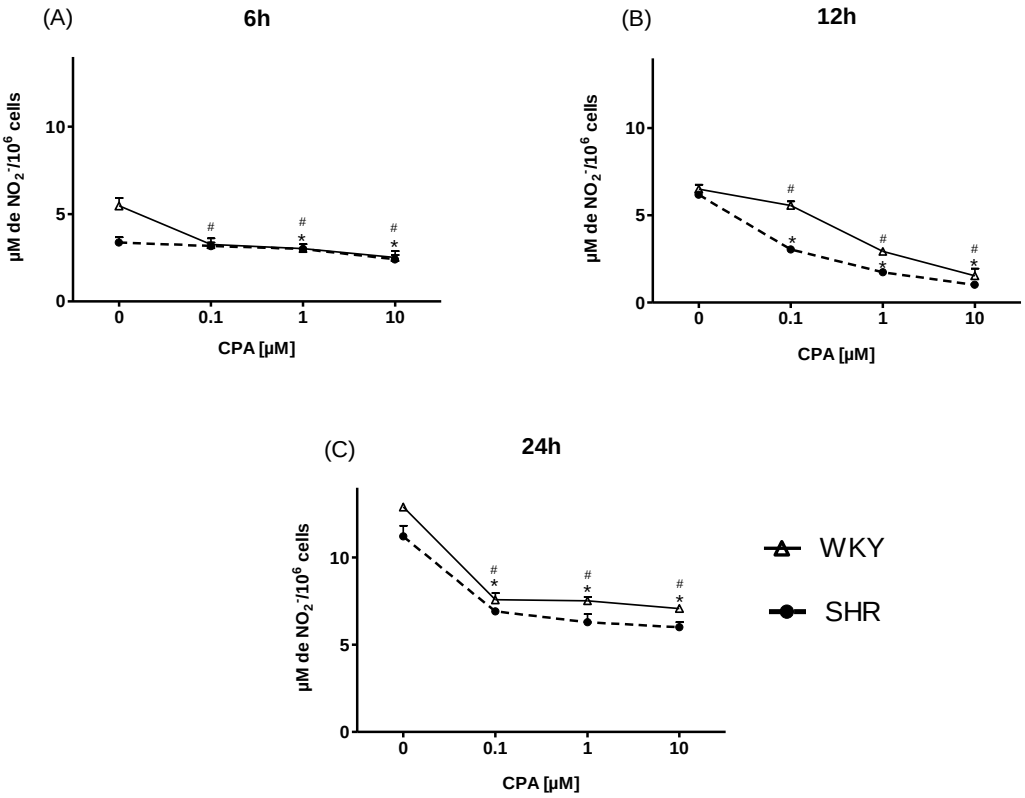


Figure 2

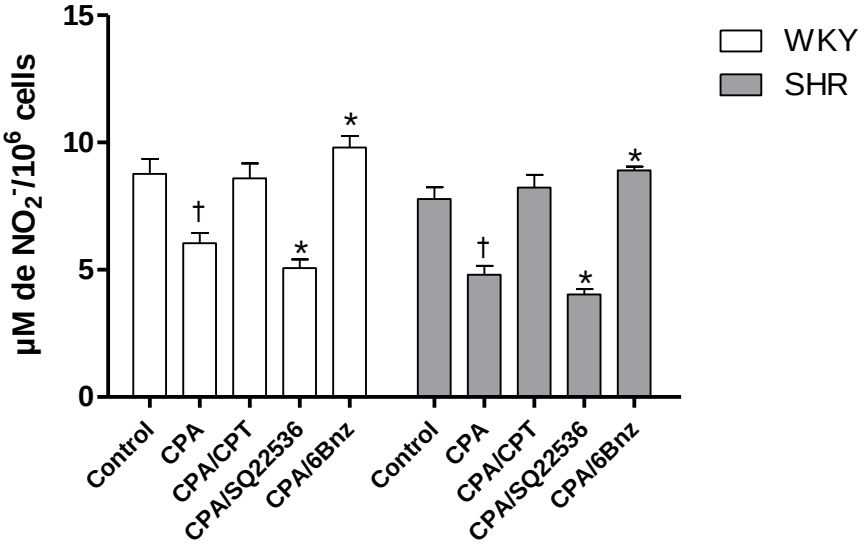
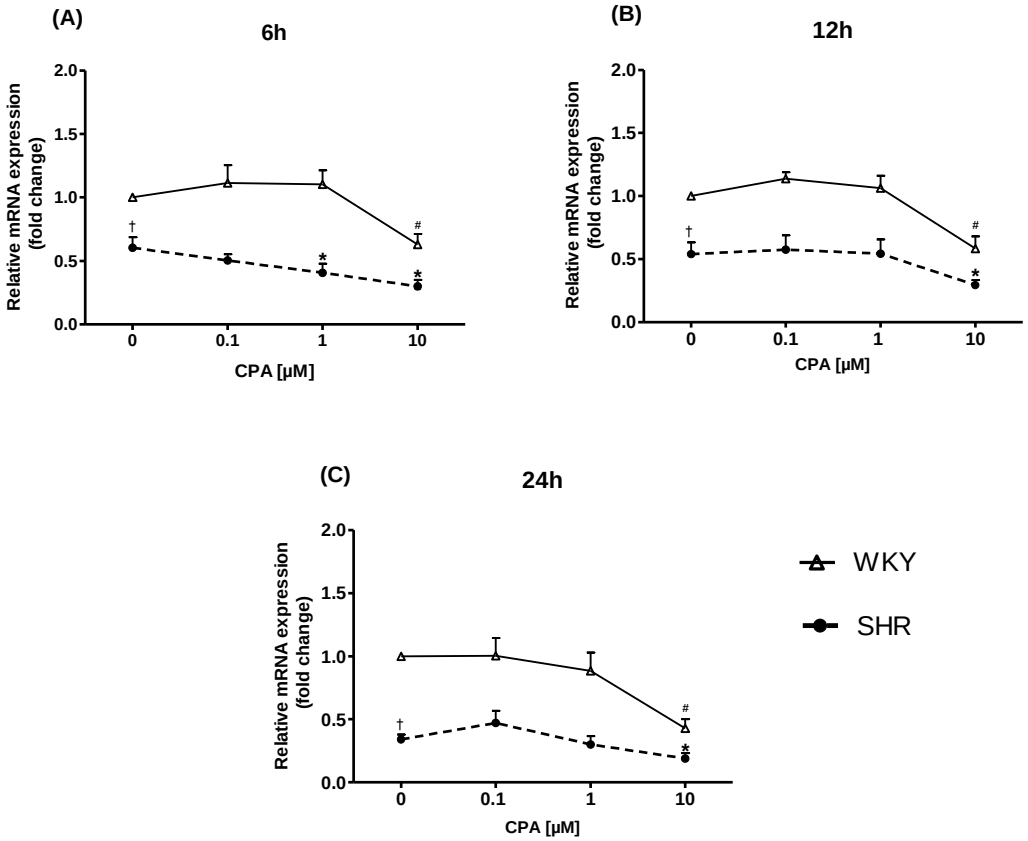


Figure 3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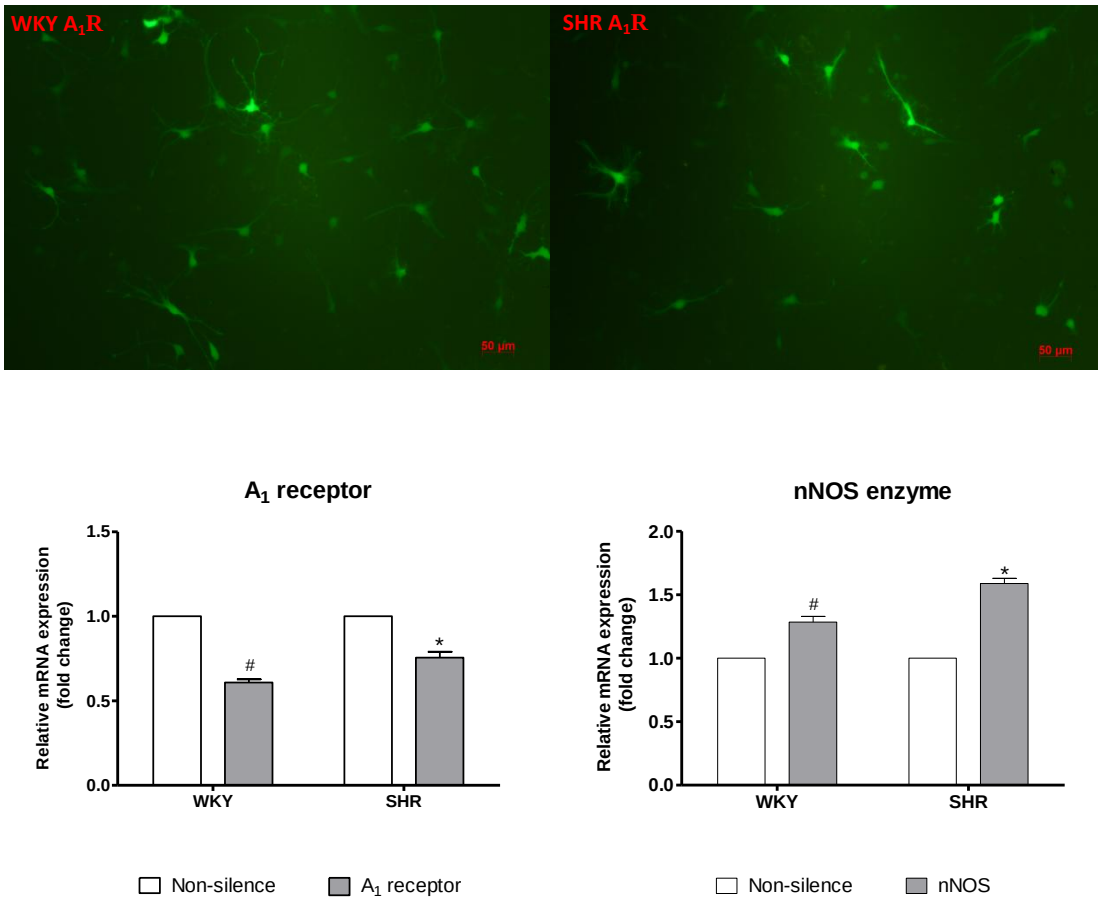
SHR

Figure 4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