Participation of opioid receptors in the cytoprotective effect of chronic normobaric hypoxia

Natalia V. Naryzhnaya[1,*], Igor Khaliulin[2], Yury B. Lishmanov[3], M.-Saadeh Suleiman[2], Sergey Y. Tsibulnikov[1], Frantisek Kolar[4], Leonid N. Maslov[1]

[1] Laboratory of Experimental Cardiology, Cardiology Research Institute, Tomsk National Research Medical Centre, Russian Academy of Sciences, Tomsk, Russia

[2] Translational Health Sciences, Bristol Medical School, University of Bristol, Bristol, United Kingdom

[3] Laboratory of Nuclear Medicine, National Research Tomsk Polytechnic University, Tomsk, Russia.

[4] Department of Developmental Cardiology, Institute of Physiology of the Czech Academy of Sciences, Prague, Czech Republic

Short Title: Opioid receptors and cardioprotection by hypoxia

*** Corresponding Author:** Natalia V. Naryzhnaya (natalynar@yandex.ru).

Laboratory of Experimental Cardiology, Cardiology Research Institute, Tomsk National Research Medical Centre, Russian Academy of Sciences, Kyevskaya 111A, 634012 Tomsk, Russia

Telephone: + 7 3822 262174

Fax: + 7 3822 555057

ABSTRACT

We studied the role of the δ, μ, and κ opioid receptor (OR) subtypes in the cardioprotective effect of chronic continuous normobaric hypoxia (CNH) in the model of acute anoxia-reoxygenation of isolated cardiomyocytes. Adaptation of rats to CNH was performed by their exposure to atmosphere containing 12% of O_2 for 21 days. Anoxia-reoxygenation of cardiomyocytes isolated from normoxic control rats caused the death of 51% of cells and lactate dehydrogenase (LDH) release. Adaptation of rats to CNH resulted in the anoxia/reoxygenation-induced cardiomyocyte death of only 38%, and reduced the LDH release by 25%. Pre-incubation of the cells with either the non-selective OR (opioid receptor) blocker naloxone (300 nM/l), the δ OR antagonist TIPP(ψ) (30 nM/l), the selective δ_2 OR antagonist naltriben (1 nM/L) or the μ OR antagonist CTAP (100 nM/l) for 25 minutes before anoxia abolished the reduction of cell death and LDH release afforded by CNH. The antagonist of δ_1 OR BNTX (1 nM/l) or the κ OR antagonist nor-binaltorphimine (3 nM/l) did not influence the cytoprotective effects of CNH. Taken together, the cytoprotective effect of CNH is associated with the activation of the δ_2 and μ OR localized on cardiomyocytes.

KEY WORDS: cardiomyocytes, chronic hypoxia, anoxia/reoxygenation, opioid receptors

1. INTRODUCTION

Acute myocardial infarction is a common cardiovascular disease with high mortality worldwide. Therefore, the protection of myocardium against ischemic injury remains a topical problem of the modern medicine. Investigation of the mechanisms underlying heart ischemic resistance that occurs naturally as a result of adaptive effects may allow us to determine the direction for the search for new cardioprotective pharmacological interventions (Heusch and Gersh 2017).

To date, convincing evidence has accumulated indicating that hearts of rats exposed to chronic continuous normobaric hypoxia (CNH) become resistant to ischemia/reperfusion injury. Tajima et al. (1994) first showed that myocardium of rats adapted to CNH is better able to restore contractile function after ischemia than the non-adapted myocardium (Tajima et al. 1994) number of subsequent studies confirmed cardioprotective effects of CNH in various in vivo and ex vivo experimental settings. CNH not only improves postischemic recovery of heart function (Maslov LN et al. 2016), but it also reduces myocardial infarct size (Borchert et al. 2011; 2017; Maslov et al. 2013; Chytilova et al. 2015; Naryzhnaya et al. 2015a) and exerts cytoprotective effects against simulated ischemia/reperfusion in isolated cardiomyocytes (Borchert et al. 2011; Naryzhnaya et al. 2015b).

Although many factors and signaling pathways have been shown to play a role in CNH-induced cardioprotection, its detailed mechanism is still unclear. At present, there is reason to presume that intracellular oxygen sensors - prolyl-4-hydroxylase (Semenza 2014), active oxygen species (Szarszoi et al. 2003), intracellular kinases such as NO synthase (Alánova et al. 2015; Zaborny et al. 2009; 2012), protein kinase C (Hlavackova et al., 2010), p38 kinase (Milano et al. 2010; Ravingerová et al. 2007), 5'-adenosine monophosphate-activated protein kinase (Sukhodub et al. 2007) participate in the mechanism of cardioprotection at chronic hypoxia. As a result of the work of these enzyme cascades, transcription factors are activated: HIF-1, nFkβ and AP-1 (Semenza 2009), changes in the functioning of mitochondrial ion channels and mPTP, which leads to improved energy metabolism of mitochondria (Maslov et al. 2015) and their resistance to ischemia-reperfusion.

Importantly, it has been established that the infarct size-limiting effect of CNH is mediated by activation of opioid receptors (OR), particularly δ_2 and μ subtypes (Maslov et al. 2013). We have shown earlier that these ORs also play a role in CNH-induced protection of the isolated perfused rat heart against ischemia and reperfusion (Maslov et al. 2015). Therefore, it can be argued that ORs, which provide an adaptive increase in heart tolerance to the impact of ischemia and reperfusion, are localized in the heart. It is known that these receptors can be located on the sarcolemma of the cardiomyocytes and on the cell membrane of endothelial cells (Maslov et al 2016). Vascular smooth muscle cells also express δ OR (Maslov et al 2016). Therefore, it has remained unclear in which cells localized ORs mediate the protective effect of CNH. The purpose of this work was to examine the role of various OR subtypes in the cytoprotective effect of CNH using isolated rat ventricular myocytes subjected to acute anoxia/reoxygenation (A/R).

2. METHODS

The experiments were carried out on male Wistar rats weighing 250–300 g. The animals were housed at 23±1°C with a relative humidity of 60–70% and a 12-h light/dark cycle with free access to water and standard rat chow. All procedures conformed to the Directive 2010/63/EU of the European Parliament and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Ethical approval was granted by the Ethical Committee of Research Institute of Cardiology, Tomsk National Research Medical Center.

2.1. Protocol of adaptation to hypoxia

The animals were randomly divided into two groups. Control rats were kept in standard normoxic conditions. The animals of the experimental group (n=13) were exposed to chronic continuous normobaric hypoxia (CNH) in the hypoxic chamber for 21 days as described previously (Neckar et al. 2003; Maslov et al. 2013). Concentrations of O_2 (12%) and CO_2 (0.3%) inside the hypoxic chamber were constantly maintained by the Bio-Nova-204G4R1 system (NTO Bio-Nova, Russia, Moscow) and monitored by the sensors TCOD-IR and OLC 20 (Oldham, France) via the MX32 control unit (Oldham, France), the temperature was maintained at 23±1°C. The animals were removed from the hypoxic chamber 24 hours before the start of the experiment. Normoxic animals (n=11) were kept in room air for the same period of time.

2.2. Isolation of cardiomyocytes and detection of anoxia/reoxygenation injury

Isolation of ventricular myocytes and induction of A/R were carried out as described earlier (Borchert et al. 2011; Xu and Colecraft 2009; Bøtker et al. 2018). The animals were heparinized (1500 IU, intraperitoneally) and sacrificed by cervical dislocation. After sternotomy, the hearts were quickly excised and placed in the Tyrode buffer (4°C) until stopped. The aorta was cannulated and fixed for retrograde (Langendorff) perfusion. The perfusion rate throughout the entire procedure was 10 ml/min, the temperature was 37°C, and all solutions were pre-saturated with 100% O_2. Hearts were perfused for 3 min with Tyrode buffer (mM/l): 140 NaCl, 5.4 KCl, 1 Na_2HPO_4, 1 $MgCl_2.6H_2O$, 10 glucose, 5 HEPES, 1 $CaCl_2$; pH 7.4. This was followed by perfusion with calcium-free Tyrode buffer for 3 min. Subsequent perfusion was performed with a solution containing (mM/l): 140 NaCl, 5.4 KCl, 1 Na_2HPO_4, 1 $MgCl_2 \cdot 6H_2O$, 10 glucose, 5 HEPES, 1.6 g/l fatty acid free BSA, collagenase type II 335 U/ml (Worthington) and XIV protease 0.230 g/l (Sigma) for 15-25 min until the myocardial softening. For the collagenase washout, the hearts were perfused with calcium-free Tyrode solution for 4 min. Ventricular myocardium was excised from the aorta and dispersed by stirring in 10 ml of calcium-free Tyrode buffer containing 10 mg/ml fatty acid free BSA. The resulting cell suspension was filtered through cheesecloth and precipitated at room temperature for 5 min. The supernatant was removed, the settled cardiomyocytes were diluted with calcium-free Tyrode buffer to 350-400 thousand cells in 1 ml. For stabilization, the isolated cells were incubated for 1 h at a temperature of 28°C under the 5% CO_2 flow in the MCO-5AC CO_2 incubator (SANYO, Japan).

After the incubation, the survival of the cells was monitored by staining with trypan blue. The percentage of the dead (stained) cells and the viable rod-shaped cardiomyocytes (with the ratio of length-to-width not less than 3:1) were counted, in each sample ~200 cells in total were analyzed in nonoverlapping visual fields using light microscopy at × 100 magnification (Axio Observer Z1 microscope, Carl Zeiss Surgical GmbH, Germany) (Borchert et al. 2011; Skyschally et al. 2018). The initial survival rate of 50% or more of the rod-shaped cardiomyocytes was considered suitable for the study. In the incubation medium of the cells, the activity of the marker of cardiomyocyte necrosis, lactate dehydrogenase (LDH), was determined after anoxia and after reoxygenation using Fluitest LDH-L kit (Analytical biotechnologies AG, Germany) and the spectrophotometer Infinite 200M (Tekan, Austria). The yield of LDH cells was calculated as a percentage of the total LDH activity, which was measured in pre-lyzed cardiomyocytes. For lysis, the cells were incubated for 45 min at 37°C with Triton X-100 at a concentration of 12 μl/ml, centrifuged for 1 min at 10,000 g, and the total LDH activity was measured in the supernatant as described above.

2.3. Experimental Protocol

The cells were divided into 7 groups before the induction of anoxia. One of the following antagonists was added to the medium 25 min before the onset of anoxia: the non-selective OR antagonist naloxone (300 nM) (Lasukova et al. 2014; Alexander S.P.H. 2017), the selective δ OR antagonist TIPP(ψ) (30 nM) (Martin et al. 2001), the selective δ_1 OR antagonist BNTX (1 nM) (Zeng et al. 2011), the selective δ_2 OR antagonist naltriben (1 nM) (Zhu et al. 2009), the selective μ OR antagonist CTAP (100 nM) (Devidze et al. 2008), or the selective κ-OR antagonist nor-binaltorphimine (3 nM) (Stevens et al. 2000). Another groups of cells that were not treated with any OR antagonist served as controls.

The cells of each group were divided into two sub-groups prior to the induction of anoxia. One of them was incubated in a modified Krebs buffer (anoxic buffer) containing (in mM/l): 118 NaCl, 25 $NaHCO_3$, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 5 2-deoxyglucose; pH 7.4. To prevent access of oxygen, the surface of the suspension was layered with 5 - 6 drops of mineral oil (Vander Heide et al. 1990). The cells were subjected to anoxia for 20 min at room temperature. After termination of anoxia, the cells were carefully pipetted through the oil and centrifuged for 1 min at 800 g. The supernatant was carefully removed and used to determine LDH concentration. The reoxygenation of the cardiomyocytes was carried out by placing them in the calcium-free Tyrode buffer for 30 min. At the end of the reoxygenation, cell survival was monitored and the LDH release was measured as described above. Corresponding (reference) sub-groups of the cells were resuspended in calcium-free Krebs buffer containing (mM/l): 118 NaCl, 25 $NaHCO_3$, 11 glucose, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4; pH 7.4 for 20 min at room temperature, then cells were centrifuged for 1 min at 800 g, supernatant was removed and used to determine LDH, and cells were incubated in calcium-free Tyrode buffer for 30 min. These sub-groups were used as controls for each group of cells subjected to anoxia/reoxygenation. Cell death during anoxia/reoxygenation was expressed as a percentage of controls not exposed to anoxia/reoxygenation.

2.4. Statistical Analysis

Results are expressed as mean ± SEM from indicated number of experiments. Data analysis was performed using STATISTICA 10. Two way ANOVA followed by Dunnett's Post Hoc test was used to detect statistically significant differences between the groups. The limit of statistical significance was $P<0.05$.

3. RESULTS

3.1. The effect of chronic normobaric hypoxia on anoxia/reoxygenation injury of isolated cardiomyocytes

The initial survival of rod-shaped cardiomyocytes from control normoxic rats and from rats adapted to CNH was 65% and 66%, respectively. The number of dead cardiomyocytes after reoxygenation was 51% in the normoxic group, while A/R caused the death of only 38% of the cells from the CNH group (Fig. 1). The release of LDH by cardiomyocytes from normoxic rats during anoxia and total LDH release during A/R was 43.57 mU/l mU/l and 61.25 mU/l mU/l, respectively (i.e. 174% and 182%, respectively, of control cells not subjected to A/R). In the CNH group, this marker of cell injury dropped to 32.42 mU/l after anoxia and to 49 mU/l after A/R (i.e. 142% and 138%, respectively, of control CNH cells not subjected to A/R) (Fig. 2).

These data indicate a marked cytoprotective effect of CNH.

3.2. The effects of OR antagonists on cytoprotection by chronic normobaric hypoxia

As follows from Fig. 1 and 2, the addition of OR antagonists to the incubation medium of cardiomyocytes isolated from the normoxic rats did not cause a significant change in their survival and LDH release during A/R compared to the untreated controls. In contrast, incubation of the cardiomyocytes from CNH rats with the non-selective OR antagonist naloxone resulted in a decrease of cell survival rate after A/R that did not differ from naloxone-treated cells from normoxic group (Fig. 1). As shown in Fig. 2, naloxone had no significant effect on LDH release during anoxia, but it abolished the protective effect of CNH on LDH release during reperfusion. These results suggest that the protective effect of CNH during reoxygenation (but not during anoxia) of isolated cardiomyocytes is mediated by the activation of OR.

Addition of the selective μ OR antagonist CTAP to the medium of cardiomyocytes isolated from rats adapted to CNH resulted in increased number of dead cells after A/R from 38% to 46% (Fig. 1) and increased LDH release by 40% compared to untreated CNH group (Fig. 2). The absence of the protective effect of CNH in the cells which were treated with CTAP suggests that μ OR play a role in CNH-induced cytoprotection.

The δ OR inhibitor TIPP(ψ) and the selective inhibitor of δ_2 OR naltriben attenuated the protective effect on cell viability and increased the LDH release after A/R in the CNH group compared to untreated cells (Fig. 1, 2). These changes were not observed during anoxia (Fig. 2). These data suggest that activation of δ_2 OR at reoxygenation contributes to the cytoprotective effects of CNH.

Treatment of cells with the δ_1 OR antagonist BNTX or the κ OR antagonist nor-binaltorphimine did not exhibit any significant effect on improved cell survival and reduced LDH release during anoxia and reoxygenation in cardiomyocytes from rats adapted to CNH (Fig. 1, 2).

4. DISCUSSION

It is well known that activation of ORs results in cardioprotection against acute ischemia/reperfusion injury (Maslov et al 2016; Heusch 2015; Headrick et al. 2015; Fraessdorf et al. 2015; Xu et al. 2015). Regarding the role of OR subtypes, it has been found that δ OR agonists have protective effect in the isolated rat heart (Karck et al. 2001; Maslov et al. 2006; Lasukova et al. 2014). Moreover, opioid TAN-67 increased cardiac tolerance to ischemia and reperfusion via δ_1 OR occupancy (Huh et al. 2001). We have studied the effects of μ OR activation on the rat heart tolerance to ischemia and reperfusion (Lasukova et al. 2015). Stimulation of μ OR in vivo by preliminary intraperitoneal administration of selective agonist DAMGO (0.1 mg/kg) reduced creatine kinase release from the isolated heart during reperfusion following global ischemia. Recently, Zhang et al. (2016) have shown that the selective μ_2 OR agonist endomorphin-1 increases cardiac tolerance to reperfusion after regional ischemia (Zhang et al. 2016). In addition, it has been demonstrated that the infarct reducing effect of Eribis Peptide 94 is mediated via μ OR occupancy (Gross et al. 2012).

It has been well established that ORs are one of the key links in the mechanism of transient cardioprotective effect of ischemic preconditioning (Heusch 2015; Fraessdorf et al. 2015; Xu et al. 2015; Schulz et al. 2001**).** The study of the infarct-limiting effect of preconditioning has revealed that this phenomenon is mediated by the activation of δ_1 OR, whereas δ_2 OR are not involved (Schultz et al. 1998). In contrast, others have reported the participation of κ OR, rather than δ_1 OR, in the protective effect of preconditioning (Wang et al. 2001). The involvement of δ and κ ORs in the cardioprotection induced by remote ischemic preconditioning has also been demonstrated (Surendra et al. 2013). In addition, we previously reported the involvement of μ, but not δ OR, in the antiarrhythmic effect of intermittent stress exposures (Maslov et al. 2004). Activation of μ and δ ORs is also involved in the infarct sparing effect of postconditioning in vivo (Zatta et al. 2008). Last but not least, we found that the δ_1 OR, but not μ and κ ORs, are involved in cardioprotective effect of postconditioning in vitro (Lasukova et al. 2014, 2016).

The above-mentioned studies allowed us to assume the possibility of participation of the opioidergic system in adaptation to hypoxia. Our findings that CNH of rats increased concentrations of endogenous δ and μ OR agonists met-enkephalin and endomorphins in blood plasma and myocardium support this hypothesis (Maslov et al. 2013). In addition, an increase in expression of the gene encoding δ OR was detected in the brain of mice after a 7-day normobaric hypoxia (Mayfield et al. 1996). We have found earlier that CNH protected rat hearts against post-ischemic contractile dysfunction and attenuated the depression of mitochondrial respiration and calcium retention capacity, and all these beneficial effects were dependent on the activation of ORs (Maslov et al. 2015). Moreover, the infarct size-limiting effect of CNH in vivo did not manifest following the blockade of μ or δ_2 ORs (Maslov et al. 2013). These data unequivocally indicate that the opioidergic system undergoes significant changes in the course of chronic hypoxia.

The main finding of the present study is that the blockade of δ_2 or μ ORs, but not δ_1 and κ ORs, abolished the cytoprotective effect of CNH against anoxia/reoxygenation injury of isolated ventricular myocytes (Fig.1, 2). These data support our previous finding and allow us to conclude that the cardioprotective effect of CNH is mediated through the activation of δ_2 and μ ORs located on the sarcolemma of cardiomyocytes (Maslov et al. 2013; Sobanski et al. 2014; Ventura et al. 1990).

Our present data indicating an important role of δ_2 and μ ORs in the cytoprotection induced by CNH are aparently in a contradiction with the above-mentioned reports, which analyzed the involvement of OR subtype in salutary effects of conditioning. We can assume that the differences in the role of OR subtypes in the mechanisms of various adaptive protective interventions studied on experimental models in vivo or in vitro may be due to the differences in the expression of different subtypes of OR on the sarcolemma of cardiomyocytes and on other types of cells. This hypothesis has yet to be investigated. The controversies over the involvement of different OR in cardioprotection may be also related to the fact that most of the endogenous opioids have low receptor selectivity. For example, enkephalins have a high affinity for μ and δ OR (Cox et al. 2015). Relative receptor selectivity has only been confirmed for endomorphins that have an affinity predominantly to μ OR (Feng et al. 2012). The low receptor specificity of endogenous opioids is accompanied by a similarity of molecular structure and intracellular signaling mechanism coupled to μ, δ and κ ORs (Feng et al. 2012). Nevertheless, the data accumulated to date are sufficient to consider the opioidergic system as an important player in the regulation of survival of cardiomyocytes during hypoxic and ischemic insults.

However, it remains unclear why the protective effect of CNH during anoxia does not depend on the activation of ORs, while these receptors mediate the protection of cardiomyocytes during reoxygenation. Our results are in agreement with the data of Cao et al. **(**2003) who did not find any effect of met-enkephalin and dynorphin on the death of cardiomyocytes during anoxia, whereas a pronounced dose-dependent cytoprotective effect of these peptides was detected during reoxygenation (Cao et al. 2003). Obviously, the protective effect of CNH during anoxia is due to other mechanisms not associated with ORs.

In conclusion, the results of our study indicate the involvement of μ and δ_2 opioid receptors of cardiomyocytes in the cell protection during anoxia/reoxygenation induced by the chronic continuous normobaric hypoxia.

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DISCLOSURE

The authors declare no conflicts of interest.

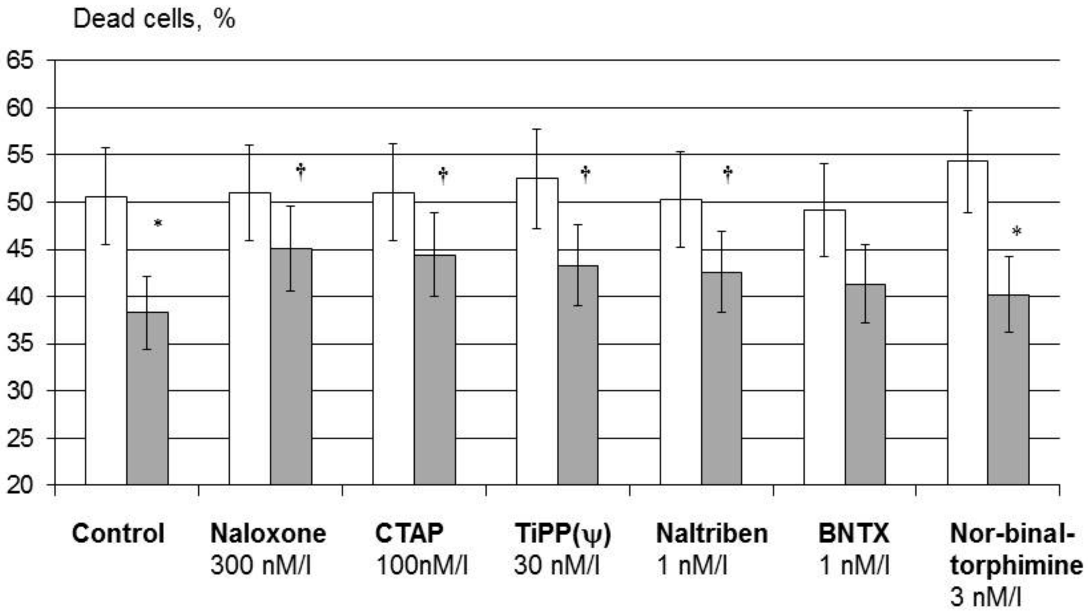

Figure 1 – Cardiomyocytes death during anoxia/reoxygenation (mean ± SEM)
Note: □ – normoxic control rats, n=11; ■ - chronic normobaric hypoxia (CNH), n=13; * $p<0.05$ – versus corresponding group of cells from normoxic rats; † $p<0.05$ – versus untreated cells from CNH rats. The difference between CNH and normoxia is not significant in BNTX group. All OR antagonists drugs were added 25 minutes before the onset of anoxia.

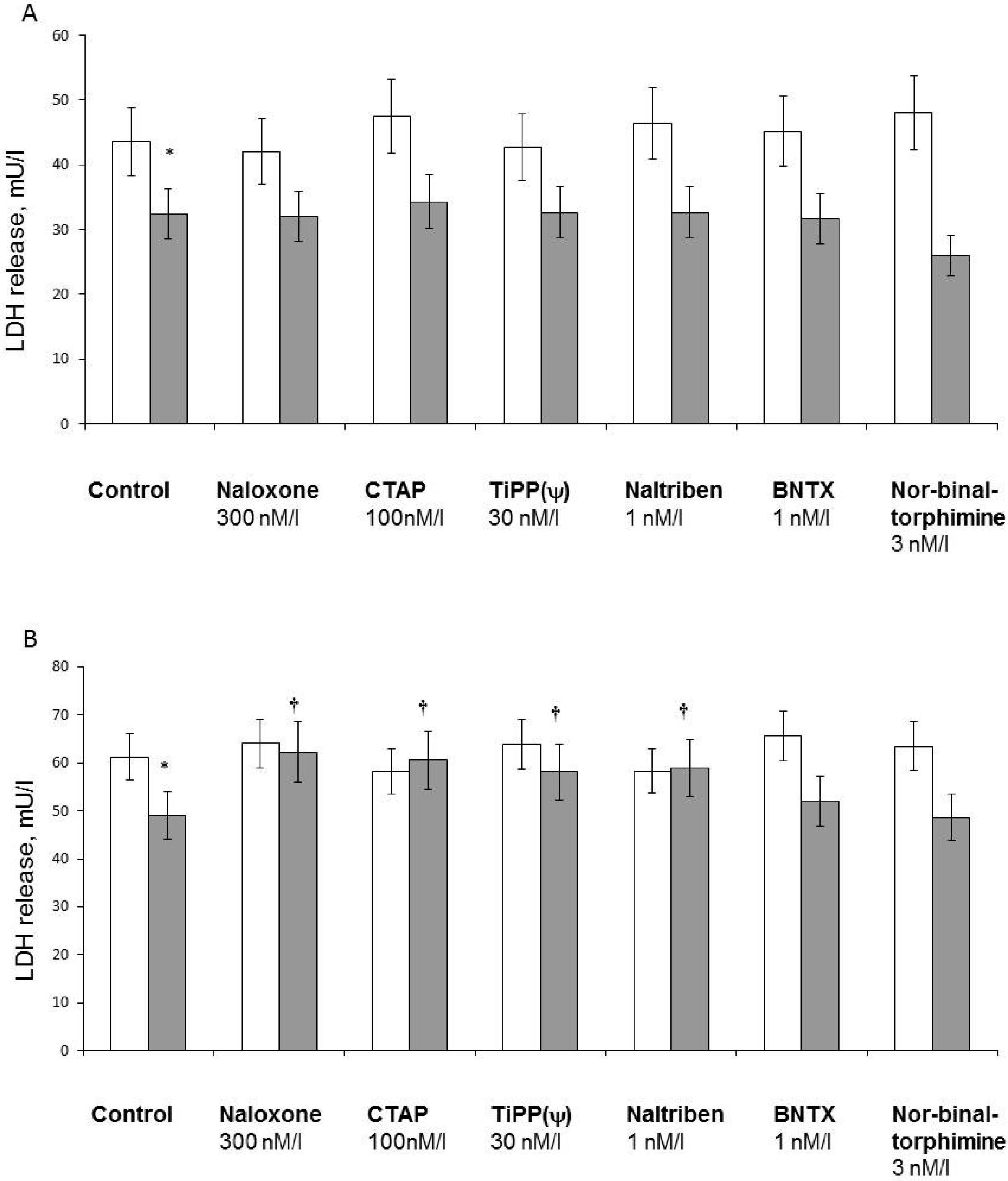

Figure 2 – Lactate dehydrogenase release from isolated rat cardiomyocytes during anoxia and reoxygenation (**mU/l**, M±SEM)

Note: **A** – Anoxia; **B** – Reoxygenation; □ – normoxic control rats, n=11; ■ - chronic normobaric hypoxia (CNH), n=13; * p<0.5 – versus corresponding group of cells from normoxic rats; † p<0.5 – versus untreated cells from CNH rats. All OR antagonists were added 25 minutes before the onset of hypoxia.

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