ORIGINAL ARTICLE

Effects of acute stressors on the expression of oxytocin receptor mRNA in hearts of rats with different activity of HPA axis

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Abstract

OBJECTIVES: Cardiovascular system is regulated by a diverse array of hormones, neurotransmitters and neuropeptides. Oxytocin and its receptors (OTR) were also shown to regulate cardiovascular functions and this hormone was even called cardiovascular hormone. In recent publication, we demonstrated the expression of mRNA of OTR by real-time quantitative PCR (RT qPCR) in all rat heart compartments. The aim of this study was to investigate the effects of acute restraint stress on OTR mRNA expression in two rat strains with different activity of HPA axis.

METHODS: Adult male Sprague-Dawley and Lewis rats, the latter strain reported to have lower HPA activity, were used in RT qPCR studies and Wistar rats in immunofluorescent ones. Both acute restraint (IS) and this stress combined with the immersion of rats in water (ICS) lasted 60 min. Gene expression of OTR mRNA was estimated in all heart compartments after 1 or 3 hours after stress termination (IS1, IS3, ICS1, ICS3). The relative expression was calculated using $2^{-\Delta\Delta C_T}$ method. In immunofluorescent studies we used commercial specific OTR antibodies.

RESULTS: In RT qPCR studies we found higher expression of OTR mRNA in atria than in ventricles and no statistical differences between Sprague-Dawley and Lewis rats under basal conditions. Relative expression of OTR mRNA after 60 min lasting stress exposure differed in dependence on the stress type and partly on the time interval after the stress termination. When compared to controls, in rat left atria both stressors caused inhibition of OTR mRNA expression in both rat strains. In rat ventricles, which have very low OTR mRNA expression, there was a significant difference in the effect of two stressors. In most groups ICS displayed the increase of OTR mRNA expression if compared to IS groups. Immunofluorescent studies revealed changes induced by acute restraint stress in all heart compartments. The immunofluorescent studies suggested that acute

stress induces higher colocalization of OTR with the nuclei than it was observed in the controls.
CONCLUSIONS: The expression of OTR mRNA in all heart compartments of controls as well as after stress exposure in Sprague-Dawley and Lewis rats support the notion that OTR plays a regulatory role in the cardiovascular system and is also involved in the regulations in the heart after stress. The immunofluorescent observation that OTRs coexpress in areas of cell nuclei in certain heart compartments and after acute stress, compared to controls, requires further studies.

INTRODUCTION

Major physiological role of the neurohypophyseal nonapeptide hormone oxytocin (OT) was until recently associated with milk ejection and uterine contraction in female mammals. Recently, over the last two decades the classical concept of OT action has greatly expanded (Zingg & Laporte 2003). The widespread distribution of OT receptors (OTR) in the brain and the specific behavioral effects of endogenous and exogenously applied OT have established the peptide as a central neurotransmitter and neuromodulator. Moreover, OTRs are expressed at multiple peripheral organs including heart and large vessels (Jankowski *et al.* 1998). Extensive studies of plasma membrane receptors proved that OTRs belong to G-protein coupled receptors and that there exists only one type of OTR (Gimpl & Fahrenholz 2001). In rat heart OT receptors, OT synthesis and release were detected in both atria and ventricles (Petersson 2002). The functionality of OT in the heart has been established by its ability to induce release of atrial natriuretic peptide (Favaretto *et al.* 1997; Gutkowska *et al.* 1997). Relatively very little is known about factors that regulate the OTR expression and OT production in the heart. However, it has been reported that OT and its receptors in the heart may play a regulatory role under stress conditions (Petersson & Uvnäs-Moberg 2007; Wsol *et al.* 2009). In view of the OT involvement in modulation of stress it is plausible to expect that the hypothalamic-pituitary-adrenal (HPA) axis can be considered as one of factors influencing the OTR expression in the heart.

In the previous study (Klenerova *et al.* 2011) we demonstrated the expression of mRNA of OTRs in all heart compartments by using real-time quantitative PCR (RT qPCR). These findings contributed to the suggestion of a potential role for OT system in cardiovascular regulation. With the aim to explore the putative involvement of HPA axis activity on the rate of OTRs expression, we employed Sprague-Dawley (SD) and Lewis (LE) rats, two strains which differ in the activity of HPA axis (Sternberg *et al.* 1992). LE rats are known to have deficient activity of HPA axis (Dhabhar *et al.* 1997; Klenerova *et al.* 2002; 2007). However, in our study, which we performed in animals under basal conditions, we observed no differences in the OTR mRNA expression between SD and LE strains (Klenerova *et al.* 2011). Therefore, in an effort to further examine the influence of the HPA activity on the process of OTR mRNA expression, we investigated in the present study the expression of OTR mRNA in the heart by RT qPCR under stress conditions. Extensive studies demonstrated differences in the effects of various types of stressors. Klenerova *et al.* (2002; 2007) described several important differences in behavioral actions of acute stressors with the prevalence of either emotional or physical components of action. Both used stressors are based on the restraint/immobilization, the physical component was strengthened by immersion of animals in water (21 °C). Another important factor for stress effects is time between stress termination and decapitation.

Thus, the aim of this study was to examine effects of acute stress on the OTR mRNA expression in the heart chambers. We used two types of stressors, one with the prevalence of emotional and the other with the additional physical component of action. The stress-induced changes were evaluated in SD and LE rat strains. Previous behavioral experiments revealed higher responsiveness to stressor stimuli in LE compared to SD rats (Klenerova *et al.* 2002; 2007; Kaminsky *et al.* 2001). Further, in order to define the cell localization of OTRs, experiments estimating the gene expression were supplemented by the immunofluorescent detection of OTRs in heart tissues by their binding of the specific antibodies.

MATERIALS AND METHODS

Animals

We used male Sprague-Dawley (SD) and Lewis (LE) rat strains (Charles-River Laboratories, Sulzfeld, Germany); their average starting body weight was 216 g and 198 g, respectively. Wistar rats (WI) (b.w. 220–240 g) (Velaz, Prague, Czech Republic) were used in immunofluorescent studies. All animals were kept under conditions described previously (Klenerova *et al.* 2011), and treatment of rats was in accordance with the Declaration of Helsinki Guiding Principles on Care and Use of Animals [DHEW Publication, NHI 80-23].The study was approved by the Ethical Review Committee, First Faculty of Medicine, Charles University in Prague.

Stress procedure

Animals of both strains were exposed to two types of acute restraint (immobilization) stressors for 60 minutes (Klenerova *et al.* 2002; 2007). Restraint alone (IS) was applied by fixing forelimbs and hindlimbs of the rat with adhesive plaster; then the animal was restrained in a snug-fitting plastic-mesh. This mesh was bent to conform to the size of individual animal and a bandage fixed this shape of mesh. When combined immobilization with water immersion (ICS), restrained rats were immersed in the water bath (21 °C) in such a way that the upper 1/4 of the animal was outside of water.

After the end of exposure to stress rats were dried and returned to the home cages. Both stresses were applied for 60 min and rats were killed by decapitation 1 or 3 h after stress termination. Thus, we had these groups: IS1, IS3, ICS1 and ICS3. ICS is considered to have more pronounced physical component of action than IS (Klenerova *et al.* 2002; 2007). Control animals remained untreated and were used directly after their removal from the home cages. For immunofluorescent studies we handled the WI rats in the same way as SD and LE rats.

RNA isolation and gene expression

The procedure for RNA isolation and gene expression was identical as described in previous publication (Klenerova *et al.* 2011). Shortly: After killing of rats, the hearts were removed; all four compartments were isolated and kept at –80 °C until homogenization. Total RNA was isolated from all compartments of controls and stress-exposed animals using TRI reagent (Sigma, St. Louis, MO, USA). RNA was reverse-transcribed using Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) for 50 min at 42 °C. Single-strand cDNA was synthesized from 4 μg of total RNA. The primers were designed to amplify the sequence corresponding to nucleotides 823–1106 (forward: ATGACCTTCATCATCGTACTGG, reverse: GTGGATGAGTTGCTCTTCTTGC) of the published rat OTR cDNA sequence (Genbank Accession No. NM_012871), and to nucleotides 873–969 (forward: TTCCTTCCTGGGTATGGAATC, reverse: GTTGGCATAGAGGTCTTTACGG) of the published β-actin cDNA (Genbank Accession No. NM_031144). The RT qPCR reactions were performed as described in previous publication with the use of iQ SYBR Green Supermix (Klenerova *et al.* 2011). The quantification analysis of the data was performed by using the Optical System Software (Bio-Rad, Prague, Czech Republic). OTR mRNA was determined in all heart chambers by subtracting their threshold cycle values (CT) from CT of reference gene β-actin. The relative expression ratios (RE) were calculated using the $2^{-\Delta\Delta C_T}$ method (Livak & Schmittgen 2001). The expression level of the β-actin gene was used to normalize for differences in input of cDNA. In experiments, following the effects of various stressors as RE of OTR mRNA, we used the control values of the appropriate heart chamber as comparator.

Statistical significance was calculated by analysis of variance (ANOVA) followed by post-hoc tests; $p<0.05$ was regarded as significant.

Immunofluorescent detection of OTR receptors

Wistar rats were killed by decapitation. Hearts were excised immediately, washed in the saline and the heart compartments, namely left atrium (LA), right atrium (RA), left ventricle (LV) and right ventricle (RV), were separated. Individual heart compartments were covered with cryomount (Bamed, Ceske Budejovice, Czech Republic) and frozen in liquid nitrogen. The procedure was as described previously (Klenerova *et al.* 2011). Shortly: We prepared 7μm sections on Kryostat Leica CM1850. After blocking the nonspecific binding sites (1% BSA, 0.1% Triton X-100 and 2% normal goat serum) we performed binding of OTRs by primary antibody Rabbit polyclonal to oxytocin receptor (Abcam, Cambridge, UK; diluted 1:500) and incubated overnight at 4 °C. As secondary antibody we used Alexa Fluor 488 Goat anti-rabbit (Invitrogen), diluted 1:400. The incubation lasted for 2 h at room temperature in the dark. After drying followed covering with one drop of Vectashield with DAPI (Vector Laboratories, Burlingame, CA, USA). For observation we used fluorescent microscope Leica DM5000 with color camera Leica DFC420 C.

RESULTS

We investigated the expression of OTR mRNA in rat heart compartments in two rat strains with a different activity of HPA axis, namely in SD and LE rats. This study represents the extension of these studies for the effect of two types of acute immobilization stress, IS and ICS, the latter known to have the prevalence of physical component of action.

Table 1 compares the relative expression of OTR mRNA in four rat compartments in SD and LE rats. RE of LA in SD was used as a comparator. Two-way ANOVA did not show significant difference between rat strains (see Table 1). One-way ANOVA calculated for the individual rat strain shows the significant differences in OTR mRNA expression in individual heart compartments (for statistics see Table 1). Data in Table 1 show very low OTR mRNA expression in both rat ventricles if compared to both atria.

Tab. 1. Relative expression of OTR mRNA in four heart compartments of two rat strains.

Heart chambers	Sprague-Dawley strain* RE ± SEM (n)	Lewis strain RE ± SEM (n)
Left atria	100.027 ± 23.437 (6)[x]	105.021 ± 14.910 (6)[x]
Right atria	8.267 ± 2.069 (6)	31.341 ± 9.488 (6)
Left ventricles	0.290 ± 0.053 (6)	0.148 ± 0.012 (6)
Right ventricles	0.013 ± 0.527 (6)	0.290 ± 0.204 (6)

Data presented as RE (x100) ± SEM. In RE calculations $\Delta\Delta C_T$ from LA of SD rats were used as calibrator

*Two-way ANOVA shows that the main effect of rat strain is not significant: $F(1,40)= 0.53$; $p=0.342$. The differences between heart compartments are significant: $F(1,40)= 75.58$; $p<0.0001$.

[x]One-way ANOVA for SD: $F(3,20)= 17.15$; $p<0.0001$, Bonferroni´s *post hoc* test shows that LA is significantly different from RA, LV and RV (for $p<0.001$).

[x]One-way ANOVA for LE: $F(3,20)=31.30$; $p<0.0001$, Bonferroni´s *post hoc* test shows that LA is significantly different from RA, LV and RV (for $p<0.001$).

The effects of IS and ICS on relative expression of OTR mRNA for SD and LE rats are shown in Figures 1 and 2. The results of stress-induced changes are related to RE of controls in appropriate heart compartments. In SD rats one-way ANOVA showed significant effects of stresses in LA ($F(4,25)=3.066$, $p<0.0348$). All four stress groups showed reduced OTR mRNA expression; significant were CO vs IS1 and IS3 ($p<0.05$). Very similar effects were found in LA of LE rats. One-way ANOVA showed $F(4,25)=4.686$, $p<0.0058$. All four stress groups showed reduced OTR mRNA expression; significant were CO vs IS1, IS3 and ICS3 ($p<0.05$).

The largest differences between stress effects in SD and LE rats were observed in RA. While in SD the values of RE in three stressed groups were increased, in LE rats the same stressed groups showed a decrease. These changes in SD were not significant while in LE rats one-way ANOVA showed reduced OTR mRNA expression vs controls ($F(4,25)=2.797$, $p<0.0478$).

In rat ventricles which express very small amount of OTR mRNA when compared to atria, we observed substantial differences between the action of IS and ICS, the combined stressor exerted the stronger stimulatory effects. In LV of SD there was significant difference between IS1 vs ICS3 and IS3 vs ICS3 groups. In RV of SD both ICS groups express more OTR mRNA than the IS groups. In LV as well as RV of LE rats the effects of ICS was significantly stronger than the effect of IS.

Figure 3 shows immunofluorescent detection of OTRs in rat heart compartments using specific primary antibody to oxytocin receptor. Samples from controls of LA, RA, LV and RV are presented on the left side, while images from tissues exposed to IS are shown on the right hand side. A positive signal of OTR expression is seen on all tissue samples, however, the individual chambers reveal a different degree and appearance of localization of these signals, which are better seen on colored pictures (not shown) than on the black and white ones. In LA, LV and RV the application of IS reduced the intensity of the positive signal; in RA stress increased levels of signals. The visual evaluation of presented images does not reveal large differences in the

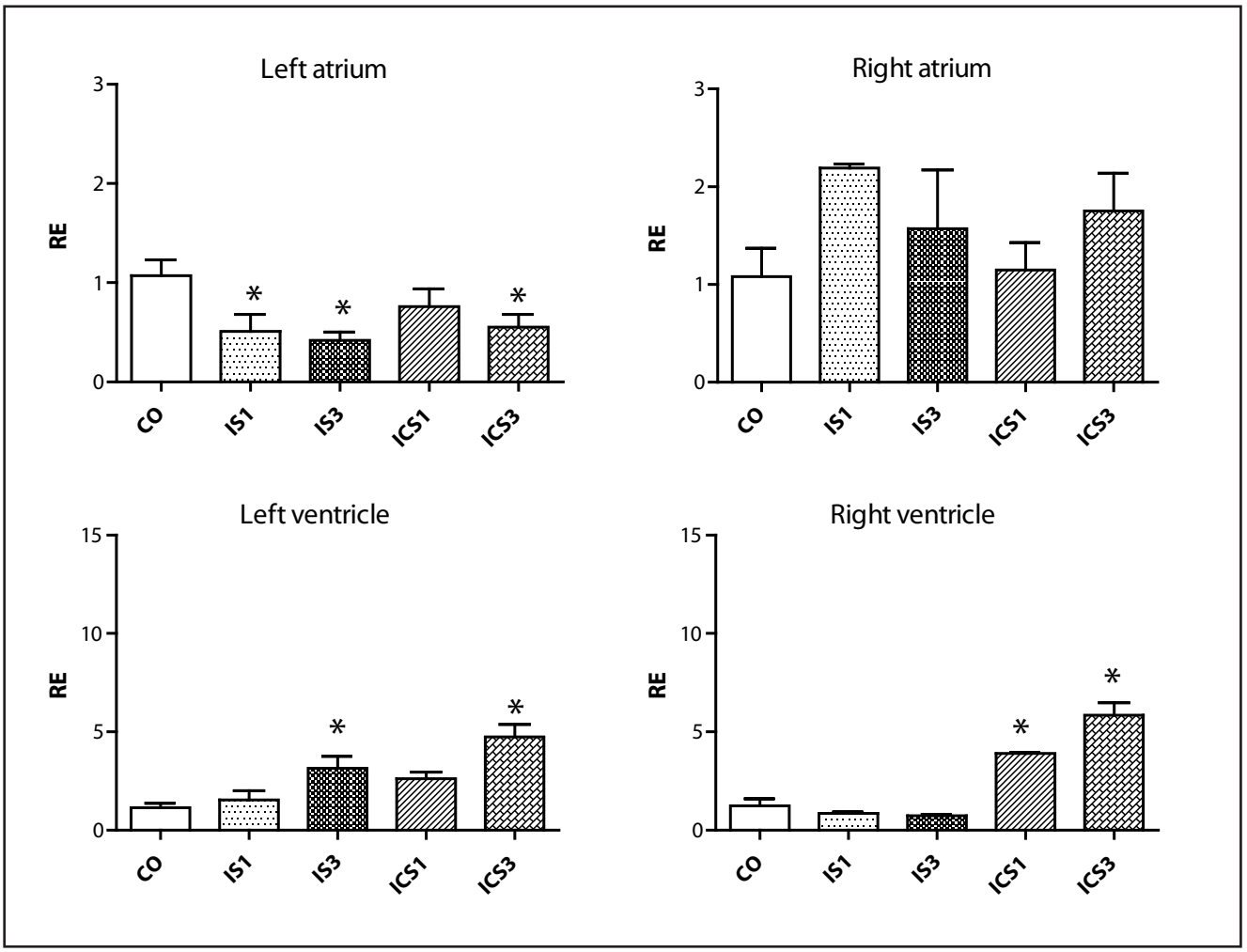

Fig. 1. Effect of acute restraint stressors on relative expression (RE) of OTR mRNA in four heart compartments of Sprague-Dawley rats. RE was calculated as described in the Methods; control values of the appropriate heart compartment were used as the calibrator. Abbreviations: IS1, IS3 are restraint stress and ICS1, ICS3 are stresses combined with immersion of rats to water; n= 6; * Statistical significance against the appropriate controls for $p<0.05$.

OTR expression in atria and ventricles. This is in contradiction with the findings of OTR mRNA expression in atria and ventricles, which express only a small fraction of the amount expressed in the rat atria.

Very interesting seems to be the translocation of the positive signals from the diffuse ones, seen in control LA, to the aggregations shown e.g. in control LV (Figure 4). The image from inserted frames is analyzed for the colocalization of OTR signals with the cell nuclei. While control tissue contains predominantly the dispersed OTR positive signal, in LV the signals are aggregated in the areas of cell nuclei.

DISCUSSION

The cardiovascular system is regulated by a diverse array of hormones, neurotransmitters and factors. Many of them are acting through stimulation of G-protein coupled receptors (GPCR). To these receptors belong also oxytocin receptors whose cardiovascular regulating role was discovered relatively recently. Substantial role in characterizing oxytocin as a cardiovascular hormone can be attributed to Gutkowska and Jankowski (Gutkowska *et al.* 2000). Nevertheless, there still remain many open questions regarding the signaling processes initiated by stimulating OTRs in the heart.

In previous investigation, exploring among others the putative influence of the HPA axis on OTR mRNA expression, we used SD and LE rat strains with differences in their HPA axis activity on the assumption that these differences might be reflected in OTR expression (Klenerova *et al.* 2011). However, estimation of the OTR mRNA expression in all heart compartments of the two strains failed to show expected differences. Since the effect of HPA activity may not be pronounced sufficiently in experiments performed in animals under basal conditions, we included in the present study investigation of the influence of two types of restraint stressors.

For the expression of OTR mRNA we used RT qPCR procedure and in calculations of RE we used values from left atria of SD controls as the calibrator.

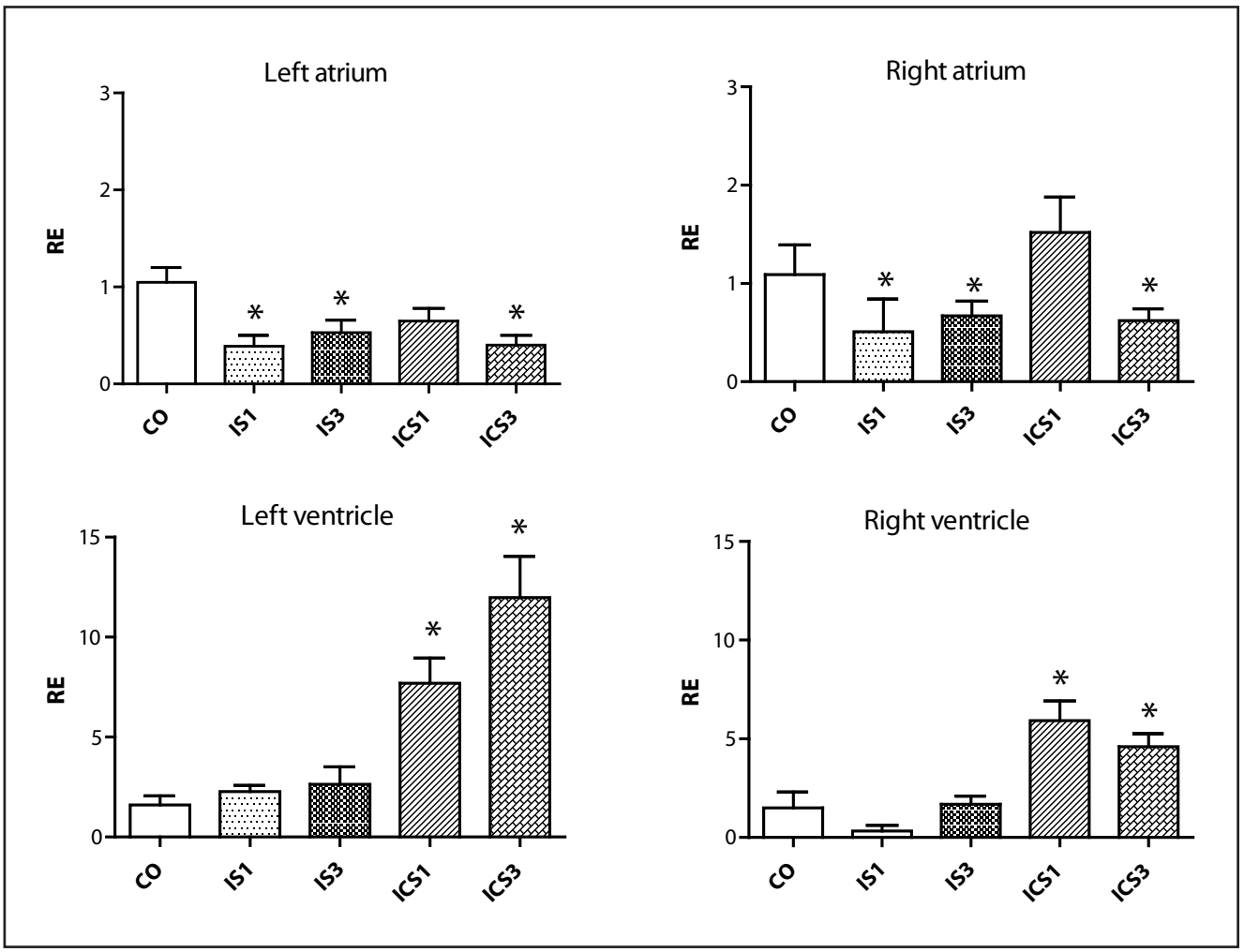

Fig. 2. Effect of acute restraint stressors on RE of OTR mRNA in four heart compartments of Lewis rats. Details as in Fig. 1.

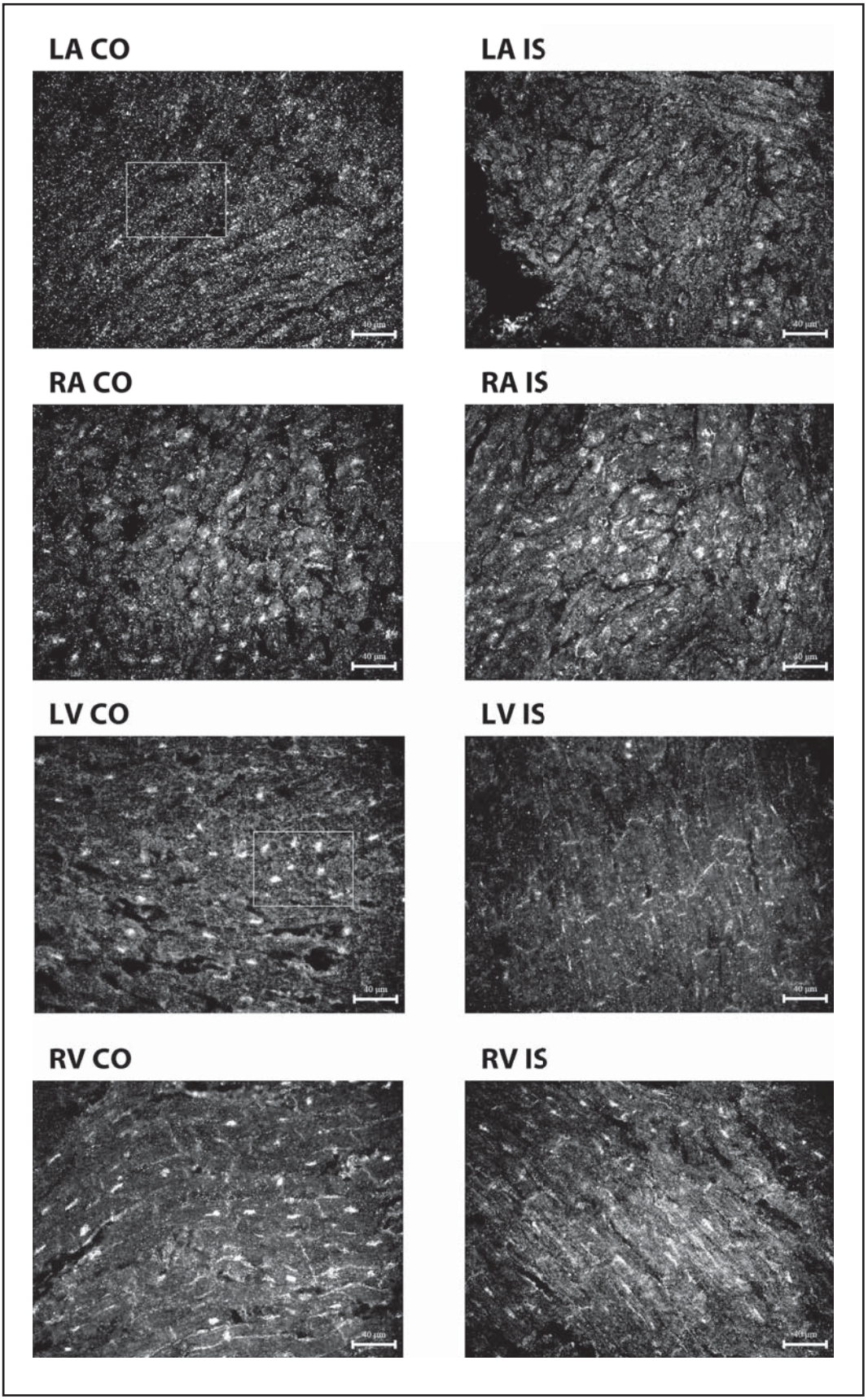

Fig. 3. Representative images of immunofluorescent detection of oxytocin receptor (OTR) signal in the Wistar rat heart. Detection by specific OTR antibodies (as described in Methods) in four heart compartments. Left side: controls, right side: IS-stressed animals. Labeled rectangles in LA–CO and LV–CO are analyzed in Fig. 4. Abbreviations: CO – controls; IS – restraint stress; LA – left atrium, RA – right atrium, LV – left ventricle; RV – right ventricle. Marker is 40 µm.

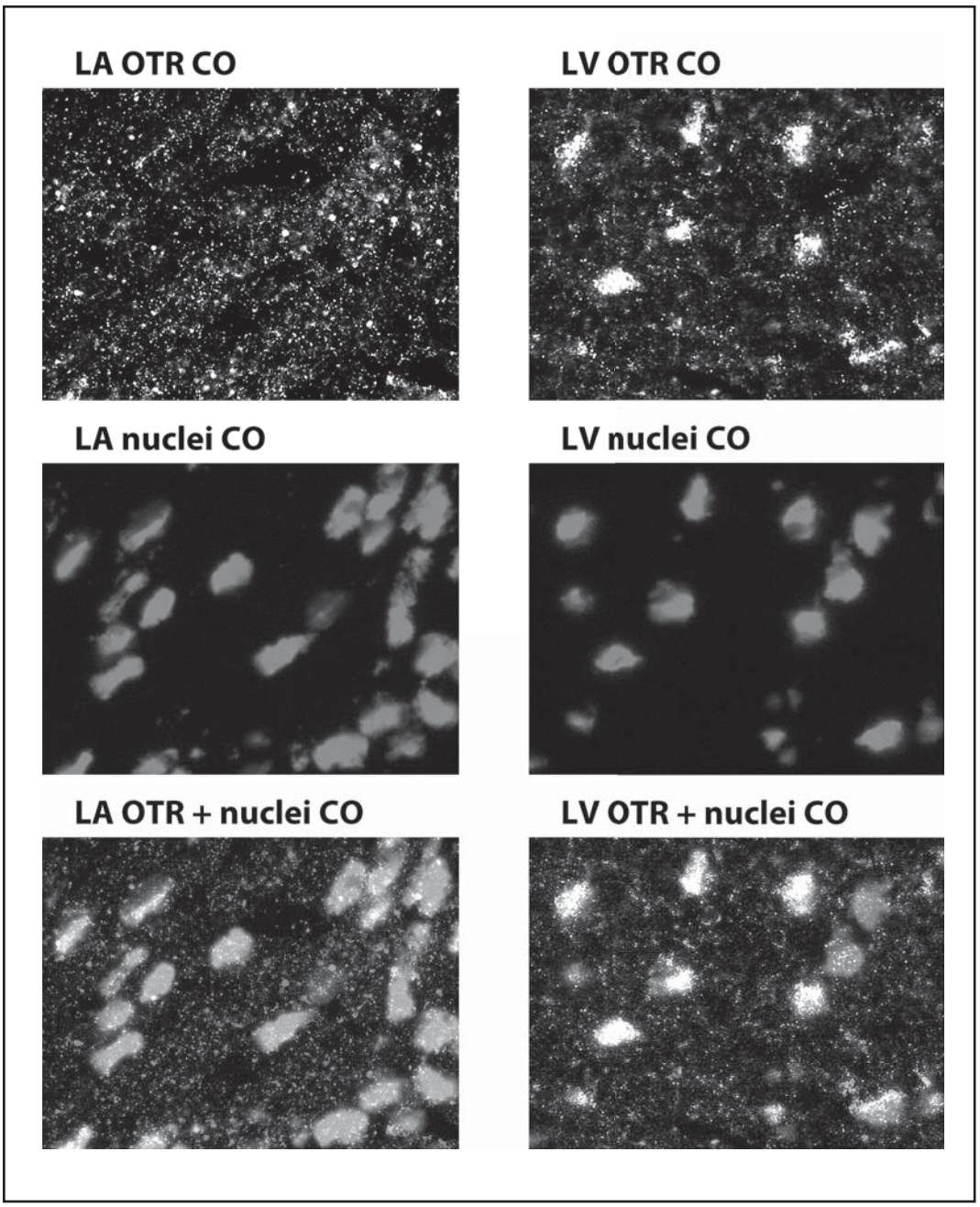

Fig. 4. Representative images from inserted frames in Fig. 3 investigating colocalization of OTR and nuclei in heart tissues. Immunofluorescent detection of OTR signal and labeling of nuclei was performed as described in Methods. Left side: left atrium from controls (LA–CO), Right side: left ventricle from controls (LV–CO). Merged images (bottom) show OTR colocalization with cell nuclei in left atria and left ventricles of control groups.

Two-way ANOVA calculations of RE of OTR mRNA in four heart compartments under control conditions did not show significant differences in SD and LE rat strains. By contrast, we observed large differences in RE values between atria and ventricles. Namely, RE in both ventricles constitutes only a small fraction of the expression in LA. These findings are in agreement with those of the group of Gutkowska and Jankowski (Gutkowska & Jankowski 2008; 2012) who came to these conclusions based on other experimental approaches (Gutkowska *et al.* 1997; 2000).

To our knowledge, no research group has investigated systematically the effects of stressors on the expression of OTR mRNA in the heart compartments. In our study we approached this problem complexly, by studying two different stressors with prevalence of emotional or physical components, which were previously thoroughly investigated in the behavioral studies (Klenerova *et al.* 2002; 2007). Moreover, we performed these studies in two rat strains with different activity of HPA axis. In these studies in each heart compartment the stress-induced values are related to RE of appropri-

ate controls. The main findings are significant effects of the stressors in both strains, suggesting the participation of OTR system in stress related responses. In left atria of both rat strains and right atrium of LE rats is significant decrease of RE after IS1 and IS3; stressor ICS evoked non significant changes. The exception in this respect represent RE values in the right atria of SD animals, where we recorded an increased detection. In ventricles, where the OTR mRNA expression forms only a small fraction of the amount found in atria, the stress-induced changes were more consistent: the RE values tend to increase in both strains and were more pronounced in LE rats. In the ventricles the combined restraint stressor (ICS) induced stronger effect than restraint stressor alone (IS). Changes in RE following both stressors were observed after 1 and 3 h after stress termination. This finding opens an interesting question of the rate of onset and duration of the stress-induced changes. In our observations of stress-induced alterations of both conditioned and unconditioned behavior we also observed stronger and longer lasting effect after the ICS (Klenerova *et al.* 2003). The results showing almost opposite effects of stress on the OTRs expression are surprising and difficult to interpret.

In the second part of our study, we performed immunofluorescent investigation of oxytocin receptor expression in rat heart compartments. In our previous publication (Klenerova *et al.* 2011) we have demonstrated in the immunofluorescent examination of control LA that the expression of OTR protein is colocalized neither with the neuronal tissue nor with the cell nuclei. This is typical for GPCRs, which are located in the plasma membranes. This paper presents another interesting finding concerning cell localization of OTRs. Immunofluorescent pictures of heart compartments in controls and after exposure of rats to simple restraint reveal substantial differences in the localization of OTR signals in tissues labeled with the specific primary antibody against OTRs. Although these images do not allow a quantitative analysis of OTRs detected in the compartments, the visual evaluation did not reveal large differences in the amount of OTRs between atria and ventricles, which could be seen in the analysis of OTR mRNA expression. In some heart compartments of controls and also after application of restraint stress there can be observed the aggregation of the OTR signal. Representative differences are shown in Fig. 4, where we demonstrate the differences in LA and LV. It is demonstrated that in the left atrium the dispersed OTR signal is mostly localized outside nuclei. On the contrary, in the left ventricle most of the OTR signal is colocalized with the cell nuclei. This observation is in concord with many new findings showing that several GPCRs are colocalized with cell nuclei (Tadevosyan *et al.* 2011). These receptors, localized in the nuclear membrane, may regulate distinct signaling pathways and they can even participate in the development of cardiovascular disorders. What is the role of receptors colocalized with nuclei requires further studies and it should be investigated the role of constitutive and ligand-induced nuclear localization of OTRs (Kinsey *et al.* 2007). The colocalization of OTRs with cell nuclei can probably partly explain the discrepancy between OTR mRNA expression and OTRs labeling by specific antibodies in the atria and ventricles. Further investigation of relations between immunofluorescent examination and the OTR mRNA expression are necessary also in regard to almost opposite effects of stress on the latter parameter in atria vs ventricles. These questions are gaining importance in view of the putative role of OT as cardiovascular function regulator.

CONCLUSIONS

The expression of OTRs mRNA in all heart compartments observed in SD and LE rats of controls as well as after exposure to stress support the hypothesis that OT and OTR play a regulatory role in the cardiovascular system. We also report that OTRs expression changes after the rats were exposed to stress suggest that the HPA axis activity may influence cardiovascular function via OTRs. The results also show dependence of the changes on the type/strenght of the stressor. The immunofluorescent observations that OTRs are coexpressed with cell nuclei require further studies. They also suggest that the biological responses mediated by OTRs may result from the integration of OTR extracellular and intracellular signaling pathways.

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