Univerzita Karlova v Praze

1. lékařská fakulta

Autoreferát disertační práce

Regulace receptorů spřažených s G proteiny

Studie muskarinových a β–adrenergních receptorů u M_2KO myší

Regulation of G protein-coupled receptors

Study of muscarinic and β–adrenoceptors in M_2KO mice

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Doktorské studijní programy v biomedicíně

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Disertační práce bude nejméně pět pracovních dnů před konáním obhajoby zveřejněna k nahlížení veřejnosti v tištěné podobě na Oddělení pro vědeckou činnost a zahraniční styky Děkanátu 1. lékařské fakulty.

Obsah

Souhrn (česky):

Cílem práce bylo podrobně analyzovat myši s vyřazeným M_2-receptorem (M_2KO) jednak za klidových podmínek a dále po působení stresového podnětu (chladový stres). Zaměřili jsme se zejména na roli antagonistických receptorů (adrenoceptorů) v srdci a jejich roli v adaptaci na chybějící M_2-receptor. Provedeny byly vazebné studie, studie genové exprese, echokardiografie, telemetrický monitoring srdeční činnosti, tělesné teploty a aktivity, analýza variability tepové frekvence, analýza biorytmů, analýza srdeční činnosti po podání agonistů a antagonistů muskarinových receptorů a adrenoceptorů, stanovení aktivity adenylyl cyklázy a NO syntázy, stanovení sérové koncentrace katecholaminů a genové exprese enzymů syntetizujících katecholaminy. Vyřazení M_2-receptorů vedlo ke kompenzatornímu snížení β_1 a β_2-adrenoceptorů doprovázené snížením jejich genové exprese, down-regulovány byly rovněž M_3-receptory. Chybějící M_2-receptory byly funkčně nahrazeny β_3-receptory, které byly upregulovány (nikoli kardioinhibičními M_4-receptory). Signalizace přes β_3-receptory vedla dále cestou adenylátcyklázy a nikoli NO syntázy. Všechny tyto změny byly zjištěny pouze v levé komoře, odpovědným mechanismem se tedy zdá být heterologní regulace. Mírně vyšší bazální frekvence a nižší tělesná teplota M_2KO myší podtrhuje důležitost telemetrické monitorace. I přes všechny pozorované rozdíly, celková srdeční funkce u M_2KO myší byla zachována.

Klíčová slova: M_2KO myši, adrenoceptory, muskarinové receptory, srdce, stres, adaptace

Abstract (in English):

The aim of the work was to perform in-depth analysis of M_2KO mice both at baseline and upon a challenge with a cold stress and to explore the role of opposing receptors (i.e. adrenoceptors) in adaptation to lacking M_2-receptors in the heart. We have performed receptor binding studies, study of receptor gene expression, echocardiography, telemetric monitoring of heart rate, body temperature and activity, heart rate variability and biorhythm analysis, analysis of heart rate response to the application of drugs (carbachol, atropine, isoprenaline, propranolol), assessment of adenylyl cyclase and NO synthase activity, measurement of catecholamine blood concentration and gene expression of catecholamine-synthesizing enzymes. We have found that the disruption of M_2-receptor gene caused a compensatory decrease of cardiostimulatory β_1-adrenoceptors and β_2-adrenoceptors with corresponding down-regulation of the gene expression, M_3-receptors were down-regulated as well. Missing M_2-receptors were functionally replaced by the main cardioinhibitory β_3-adrenoceptors that were up-regulated, not by cardioinhibitory M_4-receptors. β_3-adrenoceptors were found to signal through adenylyl cyclase instead of NO synthase. All these changes were found in the left ventricle only, so heterologous regulation is likely to be the responsible mechanism. Slightly higher basal heart rate and lower basal body temperature of M_2KO animals underscore the importance of telemetric measurement to avoid biased recordings. Despite all the changes found the overall cardiac function was unaltered.

Key words: M_2KO mice, adrenoceptors, muscarinic receptors, heart, stress, adaptation

1. Introduction

G protein-coupled receptors (GPCR) are one of the major receptor systems in organisms. Among others, they encompass adrenoceptors (AR) and muscarinic receptors (MR). Adrenoceptors mediate the effect of the sympathetic nervous system; in the heart, the effect of parasympathetic nervous system is mediated by muscarinic receptors. Adrenoceptors divide into two major types: α and β. α-adrenoceptors divide into α_1 and α_2 subtypes (they further subdivide into α_{1A}, α_{1B}, α_{1D} and α_{2A}, α_{2B} and α_{2C} subtypes). β-adrenoceptors divide into three major subtypes β_1, β_2 and β_3. Fourth β-AR (β_4-AR) is now considered to be only a low affinity state of β_1-AR (Kaumann et al., 2001). Muscarinic receptors can be divided into 5 subtypes (M_1-M_5). According to their prevailing signaling pattern, muscarinic receptors can be divided into two major classes: odd numbered subtypes (M_1, M_3 and M_5 receptors) and even numbered subtypes (M_2 and M_4 receptors).

In the heart, β_1-AR constitute about 70-80% of all β-AR (Brodde, 1991). Upon stimulation, Gs protein is activated and further activates adenylyl cyclase (AC). AC converts adenosintriphosphate (ATP) into cyclic AMP (cAMP). cAMP further activates protein kinase A (PKA) resulting in phosphorylation of multiple proteins including those involved in coupling between excitation and contraction. This classical paradigm is true for only intermittent or short-term β_1-AR activation. Prolonged β_1-AR stimulation leads to G protein receptor kinase (GRK)-mediated receptor phosphorylation, which leads to the recruitment of β-arrestin (β-ARR) to phosphorylated β_1-AR. β-arrestin plays two different roles in β_1-AR signaling: it works as a sterical inhibitor that blocks further interaction between the receptor and G protein and targets the receptor for internalization (Lefkowitz, 1998). Second, it serves as scaffold protein that enables the interaction of other members of signaling cascade (Mangmool et al., 2010). Prolonged β_1-AR activation leads to the activation of calmodulin activated protein kinase II (CaMKII) that is responsible for clinically unfavorable effects of β_1-AR activation (i.e. increased apoptosis) that is observed in untreated heart failure (Yoo et al., 2009). However, signaling through β_1-AR can have a protective role as well as it has been showed that β_1-AR and β-arrestin can also signal through matrix-metalloproteinase (MMP)-mediated cleavage and extracellular shedding of heparin-binding epithelial growth factor (HB-EGF) that ultimately leads to EGFR transactivation (Noma et al., 2007), which confers cardioprotection.

β_2-AR constitute only about 20-30% of total β-adrenoceptors in the heart (Brodde, 1991). Although β_2-AR couple to Gs protein (similarly to β_1-AR signaling), they are also able to couple to Gi, which elicits the contrary effect. β_2-AR coupling to Gi is preceded by PKA-

mediated phosphorylation of β_2-AR. Both β_1-AR and β_2-AR work usually in a complementary manner; however, under certain circumstances their function might be opposing. It has been showed that the stimulation β_1-adrenoceptor promotes apoptosis (Zhu et al., 2003) whereas the stimulation of β_2-adrenoceptor has anti-apoptotic effects (Chesley et al., 2000).

The presence and function of β_3-AR is still a matter of debate. The activation of β_3-AR can produce different effects depending upon the heart chamber- atria vs. ventricles (Brixius et al., 2004) and upon species (Skeberdis et al., 2008). In human ventricles, stimulation of the β_3-AR produces a marked decrease in cardiac contractility (Gauthier et al., 1996), which is in contrast to the other β-AR subtypes. It has been supposed that β_3-AR serves as a „brake" during excessively increased sympathetic overstimulation since it is activated at high catecholamine concentrations and counteracts the effects of β_1 and β_2 activation by producing a negative inotropic effect (Moens et al., 2010). On the other hand, it has been showed that in human atria β_3-AR stimulation increases contractility (Skeberdis et al., 2008). In human ventricles, the activation of β_3-AR causes an activation of the nitric oxide (NO) pathway. Recent data suggest that all three NO isoforms (eNOS, nNOS, iNOS) are involved (Brixius et al., 2004, Amour et al., 2007, Maffei et al., 2007).

Finally, a fourth β-adrenoceptor (β_4-AR) was described, but it was found later that this β_4-AR is only a low affinity state of β_1-AR (Kaumann et al., 2001). The current concept of signaling through β-receptors is the figure in the middle of the booklet.

In the heart, the dominant muscarinic receptor subtype is M_2. When stimulated, M_2-receptors activate Gi protein that inhibits adenylyl cyclase and thus counteracts cAMP-PKA-dependent signaling pathways. Besides M_2-receptor, there is also a population of non-M_2-receptors, but the exact nature of these receptors is still a matter of debate. Binding studies performed on rats suggested that the minor subtype comprise of M_1- and M_5-subtype (Myslivecek et al., 2008). However, different studies (on mice) suggested that the major non-M_2-subtype were M_1, M_3 and M_4 (Wang et al., 2004).

Both adrenoceptors and muscarinic receptors were proved to couple to multiple G proteins and even signal through G protein independent pathways, switch their signaling pathways over time and interact within or between their receptor subfamilies (Nathanson, 2000, Xiao et al., 2006). The paradigm of linear G protein-coupled signaling has gradually yielded to the model of „signalome", where an individual adrenoceptor associates with multiple signaling pathways in time and spatially specific manner (Park and Palczewski, 2005).

In the past decades, knock-out organisms have been widely used to study physiological and pathological processes. To better characterize the role of adrenergic and muscarinic receptors, multiple knock-outs have been prepared and investigated; i.e. mice with the knock-out of M_2-receptors (Stengel et al., 2000), β_1-adrenoceptors (Rohrer et al., 1996, Ecker et al., 2006), β_2-adrenoceptors (Chruscinski et al., 1999, Ecker et al., 2006), β_3-adrenoceptors (Varghese et al., 2000), β_1/β_2-adrenoceptors (Kiriazis et al., 2008) and $\beta_1/\beta_2/\beta_3$-adrenoceptors (Lee et al., 2010). However, only a few papers have described the alteration of counter-acting receptor responses in knockout animals (LaCroix et al., 2008). The focus of the thesis was to describe changes in adrenergic and muscarinic receptors in the heart upon M_2-receptor knock-out. In order to obtain better insight into processes that compensate for missing M_2-receptors we challenged animals with a stressor (cold stress).

2. Goals and Hypotheses

Despite M_2KO mice were already a matter of investigation (Seeger et al., 2004, LaCroix et al., 2008), very little is known about the adaptation mechanisms that take place after the knock-out of M_2 gene. Similarly, the way they cope with stress is not well understood either.

The goal of the work was to perform in-depth analysis of M_2KO mice both at baseline and upon a challenge with a cold stress and to explore the role of opposing receptors i.e. adrenoceptors in adaptation to missing M_2-receptors. We have focused on the receptor level, subsequent changes in intracellular signaling as well as on the consequences in physiological functions on the whole-organism level.

We have hypothesized that:

1. There would be a down-regulation of cardiostimulatory β_1- and β_2-adrenoceptors based on the down-regulation of the β_1 and β_2-adrenoceptor gene expression. Since β_3-adrenoceptors have been proved to mediate cardioinhibitory function we have assumed that these receptors would be one of the receptors employed to overtake the missing function of M_2.
2. M_4-receptors would possibly take over the function of missing M_2. Other non-M_2-subtypes would not be significantly altered.
3. There would be no difference in the systolic and diastolic function of M_2KO hearts.
4. There would be no difference in the heart rate, activity and basal temperature.
5. There would be no difference in the level of circulating catecholamines and the activity of catecholamine synthesizing enzymes.
6. The condition of prolonged (one week) stress would lead to different adaptation responses in M_2KO mice with distinct adjustment in cardiac signaling.

3. Material and Methods

Experimental animals: M_2−/− (M_2KO) mice (genetic background: 129 J1× CF1, generated as described previously (Gomeza et al. 1999)) were used for experiments. WT mice (M_2+/+, 129 J1×CF1) were used as controls. Animals were treated in accordance with the legislature of the Czech Republic and the EU. The animals were maintained under controlled environmental conditions (12/12 light/dark cycle, lights on at 6 AM). Food and water were available *ad libitum*. The average ambient light phase temperature±SEM was 24.45±0.4 °C; the average ambient dark phase temperature±SEM was 24.22±0.4 °C. During cold stress, animals were exposed to cold for 1 day (24 h) or 7 days in conditions simulating natural situation. In the biological night (from 8 p.m. to 8 a.m.) the animals were exposed to the temperature 5.91± 0.38°C, while in the biological day the temperature rose to 9.73± 0.59°C.

Receptor binding: Receptor binding sites were determined as follows: total β_1-AR + β_2-AR binding sites using [^{3}H]-CGP 12177, β_3-AR using [^{3}H]-SB 206606, MR using [^{3}H]-QNB. Non-specific binding was determined with 50μmol/l propranolol (for total β-AR), 1mmol/l SR59230A (for β_3-AR), 5μmol/l atropine for MR. Incubation times were performed as published previously (Myslivecek et al., 2007). Affinity constants (K_D) were used for the "single-point" measurements. 2,000 pmol/l [^{3}H]-CGP 12177, 120 nmol/l [^{3}H]-SB 206606 and 1,500 pmol/l [^{3}H]-QNB were used. β_1- and β_2-AR were determined using co-incubation with 400 pmol/l [^{3}H]-CGP12177 and subtype-selective antagonists (10^{-7}mol/l CGP 20712A for β_1-AR and 10^{-8}mol/l ICI 118.552 for β_2-AR).

Receptor gene expression: Total mRNA was isolated using the chloroform–isopropanol method. After RNA purification and reverse transcription into cDNA, qPCR was performed using TaqMan probes. The levels of the analyzed transcripts were normalized to the level of the reference gene transcript (18SrRNA, the level of which was practically the same in WT and KO).

Echocardiography: Echocardiography was done as published previously (Tanaka et al., 1996). Interventricular septum thickness, posterior wall thickness, left ventricle end-systolic, end diastolic diameters, cycle lengths, mitral valve E and A wave velocities, E/A ratio, mitral valve deceleration time and slope, maximal velocity in the left ventricle outflow tract and left ventricle ejection time were measured in the appropriate positions.

Telemetric assessment of heart rate, body temperature and animal activity: The transponders (E-Mitter, G_2-HR) were implanted in the peritoneal cavity and mice were left for 1 week to recover from the surgery and then used in experiments. The sensor leads were used for heart

rate data acquisition; other parameters (temperature and activity) were acquired directly from the transponders. Heart rate and temperature was collected every 10s, activity every 60s.

Drug application: The application route was intraperitoneal (i.p.) and approximately 100µl (according to the weight) was applied to each animal. All of the drugs were administered at the same daytime (9:30 AM to 9:45 AM). Carbachol (carbamoylcholine chloride, 0.5 mg/kg), isoprenaline (isoprenaline hydrochloride, 50 mg/kg), atropine (atropine monohydrate sulfate, 30 mg/kg), and propranolol (propranolol hydrochloride, 20 mg/kg) were applied.

Adenylyl cyclase (AC) activity (basal, forskolin-stimulated, β_3-adrenoceptor stimulated): AC activity was determined similarly as published previously (Hoffert et al., 2005). The incubation with forskolin (40 µmol/l) and CL 316243 (β_3-agonist, 100 nM) were used to determine stimulated AC activity and the effect of β_3-AR activation.

Determination of nitric oxide synthase (NOS) activity: NOS activity was determined using Ultrasensitive Colorimetric NOS Assay Kit (Oxford Biomedical Research, Inc., USA). BRL 37344 (1 mM) was used as specific β_3-agonist.

Catecholamine blood concentration: Catecholamines were determined by enzyme immunoassay using 2-CAT Research ELISA kit DEE5400 (Demeditec Dg., Germany).

Gene expression of catecholamine-synthesizing enzymes: Total RNA was prepared from frozen mice adrenal medullae using the RNAzolTM reagent (Tel-Test), according to the manufacturer's instructions. After reverse transcription reaction (using Ready-To-Go You-Prime First-Strand Beads) PCR reaction was performed as published previously (Benes et al., 2012). Semiquantitative values were expressed relative to the housekeeping gene (GAPDH).

Statistical analysis: Statistical differences among groups were determined by 1-way analysis of variance (ANOVA). For multiple comparisons, modified Newman–Keuls correction was used. The data from drug application experiments were evaluated using 2-way ANOVA (repeated measures; factors: treatment, phenotype).

4. Results

We have initially performed binding experiments and subsequently gene expression experiments both in the left and right ventricles. In right ventricles, no difference in binding and gene expression in all analyzed muscarinic and β-receptor subtypes were found between WT and KO animals except for binding of muscarinic receptors that was diminished to the similar extent as in left-ventricle (6% of WT). Therefore, other signaling parameters (adenylyl cyclase, NO-synthase) were analyzed in left ventricles only. All data presented below thus pertain to the situation in left ventricles.

4.1. Binding characteristics, basal state

M_2–knockout changed the amount of all β_1-, β_2-, and β_3- adrenoceptor binding sites (Fig. 4.1.1). The cardiostimulatory β_1- and β_2-AR were decreased but the number of β_3-AR were increased in M_2KO mice. The binding of muscarinic receptor specific radioligand in M_2KO mice was not completely abolished with 13 % of remaining binding sites.

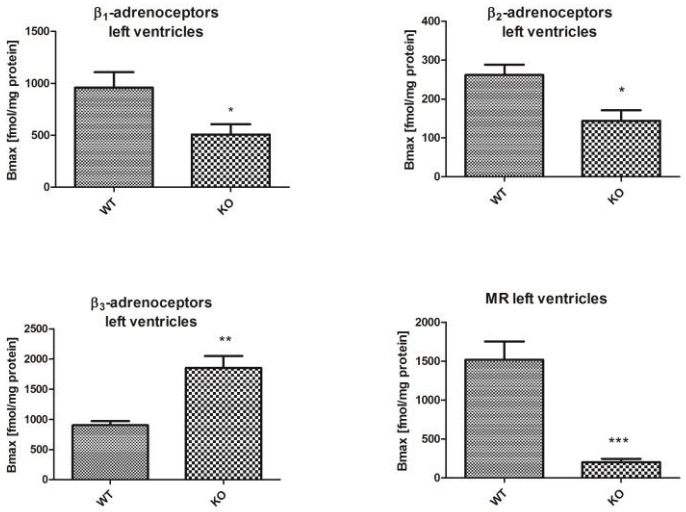

Fig. 4.1.1. The changes in the number of β_1-, β_2-, β_3-AR and MR binding sites in M_2KO mice.
*p<0.05, **p<0.01 different from WT, ***p<0.001 different from WT, n= 3–4 (β_1- and β_2-AR) and n= 6 (MR and β_3-AR)

The experiments with saturation binding and competition with CGP20712A and ICI118.151 proved that the affinity of receptors (total β-AR, MR, β_3-AR) was not affected by M_2 muscarinic receptor knockout, which suggests that observed changes occur in the amount of binding sites rather than in the affinities of receptors to ligands.

4.2. Receptor binding upon stress

In WT animals, the cold stress resulted in a decrease in β_1- and β_2-AR (both after 1 day stay in the cold and after 7 days in cold) and also in a decrease in MR. β_1-AR decreased to 37% of control values after 1 day of stress and further decreased to 27% of control values after 7 days of cold, β_2-AR were diminished to 35% after 1 day of cold and to 28% after 7 days in cold. On the contrary, β_3-AR increased (to 216% of control values) after 7 days of cold. Muscarinic receptors decreased to 46% of control values after 1 day of cold and the changes prevailed till the day 7 (when 58% of control values were measured), Fig. 4.2.1.

The reaction of cardiostimulatory β_1- and β_2-AR to cold was similar in KO animals. In KO animals, there was a decrease in β_1-AR number after 1 day of cold (to 45% of KO control values) and further persisted- 32% of KO control values were measured after 7 days of cold. The changes in β_2-AR were almost the same as those in WT: β_2-AR decreased to 44%

and to 29% in KO animals (after day 1 and day 7, respectively). In contrast to cardioinhibitive receptors in wild types, β_3-AR in KO animals (which were already increased at baseline compared to WT) did not further change in reaction to cold. Muscarinic receptors (that were greatly attenuated at baseline in KO) did not further change upon cold stress, Fig. 4.2.2

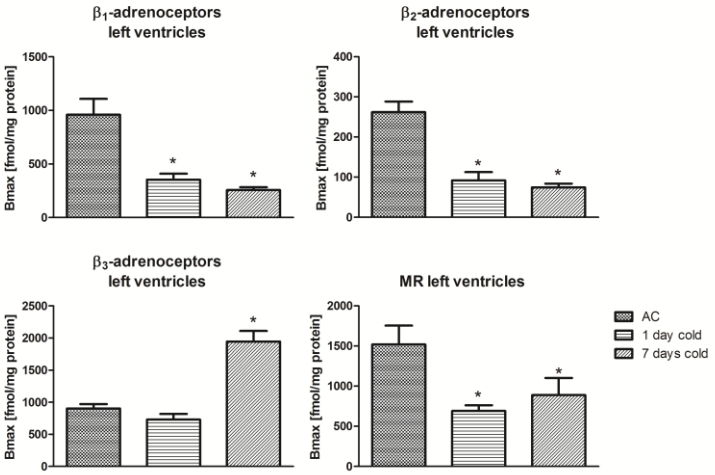

Fig. 4.2.1. Changes of receptor binding during exposure to cold in wild type animals in left ventricles.

Abscissa: number of days in cold. Ordinate: receptor binding expressed as B_{max} [fmol/mg. prot.$^{-1}$].

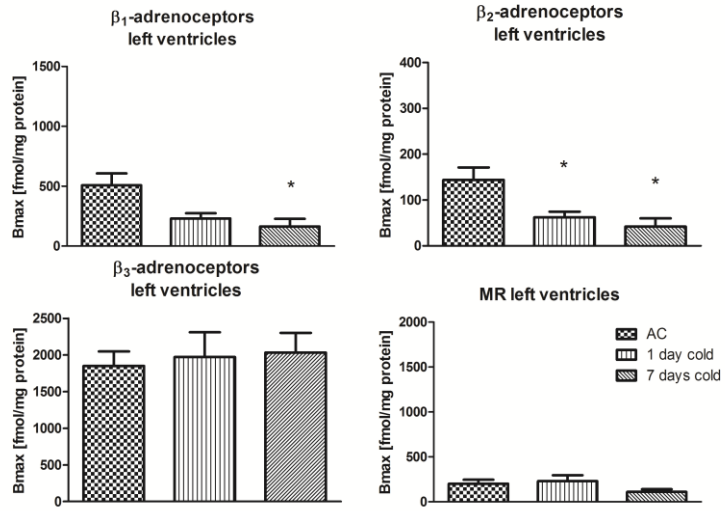

Fig 4.2.2: Changes of receptor binding during exposure to cold in knockout animals in left ventricles.

Abscissa: number of days in cold. Ordinate: receptor binding expressed as B_{max} [fmol/mg. prot.$^{-1}$].

4.3. Receptor gene expression, basal state

M_2-MR were naturally not expressed in KO animals, moreover M_1-MR and M_5-MR were not detected both in WT and KO mice. M_3-MR gene expression was diminished in KO while M_4-gene expression remained unchanged. The gene expression of both β_1-AR and β_2-AR was decreased to approximately 60 % in KO animals (Fig. 4.3.1.).

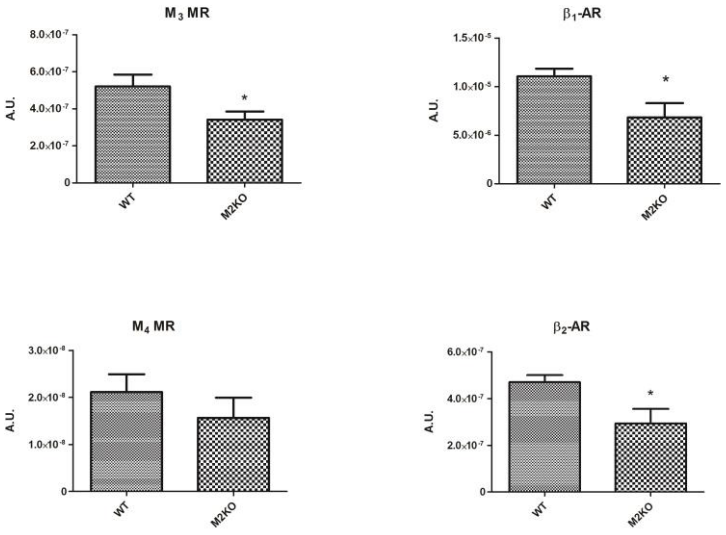

Fig. 4.3.1. The changes in M_3, M_4, and β_1- and β_2-adrenoceptor gene expression in left ventricles in M_2KO animals.

*p<0.05 different from WT, n=8 (WT and KO). M_2-MR receptor gene expression was undetectable in KO mice; M_1-MR and M_5-MR gene expressions were undetectable both in WT and KO mice.

4.4. Receptor gene expression upon stress

Upon cold stress, the expression of M_4-MR was not changed both in WT and KO, while the expression of M_3-MR decreased in WT but not in KO (to 52% after 1 day of stress and to 42% after 7 days of stress).

4.5. Echocardiographic parameters

The echocardiographic parameters describing both LV systolic and diastolic function were similar both in WT and KO animals.

4.6. Heart rate

When comparing means in WT and KO animals as averaged in 30-min intervals, we have found a 6% increase in basal heart rate in M_2KO animals. The difference between animals was also seen when comparing light phase and dark phase means. Both WT and KO animals displayed a typical circadian pattern in HR with peak values in the dark span, Fig. 4.6.1.

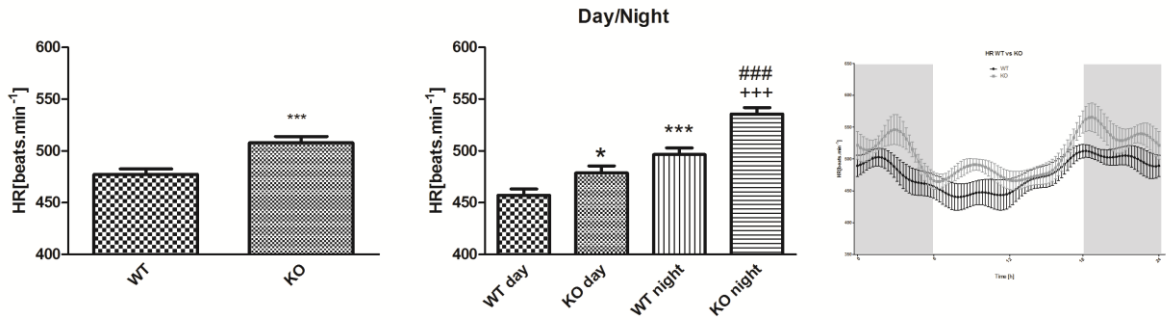

Fig. 4.6.1. Heart rate in WT and M_2KO mice.

Average heart rate measured in 30-min intervals over a 24-hour period (left). Heart rate in the light and dark phase (middle). Comparison of biorhythm in WT and KO animals (right). *p<0.05 different from WT day, ***p<0.001 different from WT day, +++p<0.001 different from WT night, ###p<0.001 different from KO day.

4.7. Heart rate variability

Both animal groups (WT and KO) showed a similar distribution of power spectral densities (PSDs). When areas under the curves (AUCs) were compared, Mann–Whitney test showed no difference between groups.

4.8. Activity

Both WT and KO animals displayed a typical circadian pattern in their activity with peak values in the dark span. No difference in activity was observed both when the data were averaged in 30-min intervals over the whole 24-hours period as well as when the data from the light phase and the dark phase were treated separately. No difference in biological rhythm analysis was seen as well.

4.9. Body temperature

The body temperature in M_2KO animals was lower than in WT. When comparing the light and the dark phase, the same picture was obtained. Both WT and KO animals displayed a typical circadian pattern in temperature with peak values in the dark span. There was no difference in biological rhythm analysis (Fig. 4.9.1.).

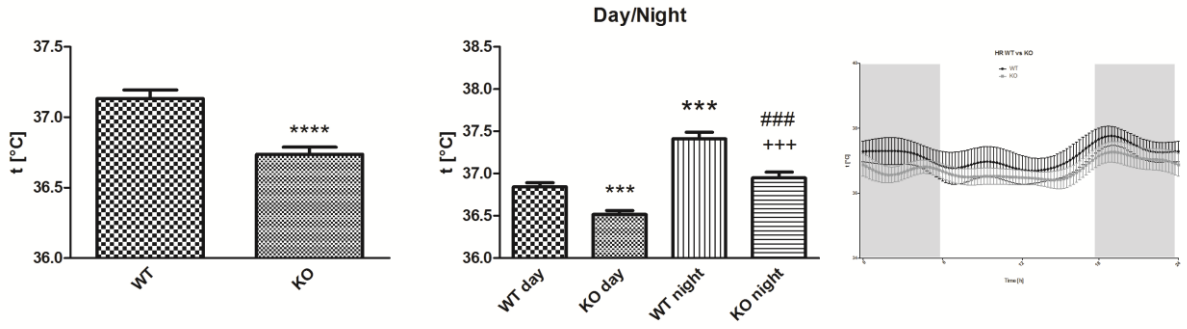

Fig. 4.9.1. Body temperature

Body temperature in WT and M_2 KO mice. Left: average temperature. Middle: temperature in the light and dark phase. Right: comparison of biorhythm in WT and KO animals.

****p<0.0001 different from WT, ***p<0.001 different from WT day, ###p<0.001 different from KO day, +++p<0.001 different from WT night

4.10. Drug application

Carbachol caused expected bradycardia in WT animals; on the contrary, it induced tachycardia M_2KO animals. Isoprenaline also caused expected tachycardia in WT. The same effects were observed in KO animals, but the effect was diminished here. Atropine increased heart rate in WT, but failed to affect the heart rate in KO mice. Propranolol significantly decreased the heart rate both in WT and KO animals, but its effect was diminished in KO (Fig. 4.10.1.).

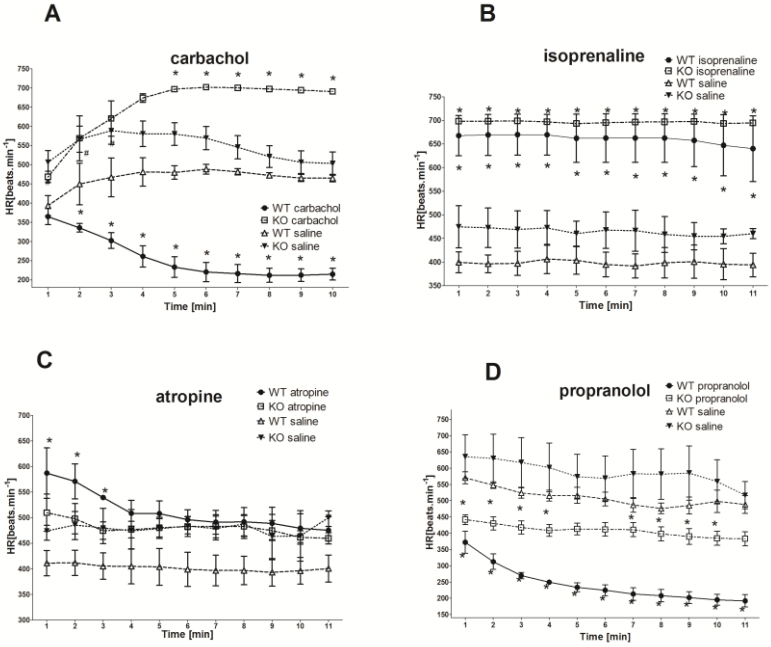

Fig. 4.10.1. The effects of carbachol (0.5 mg/kg, a), isoprenaline (50 mg/kg, b), atropine (30 mg/kg, c), and propranolol (20 mg/kg, d) on heart rate .

Abscissa, time, ordinate, heart rate [beats.min^{-1}]; *p<0.05 from saline-treated animals, #p<0.05 from WT saline-treated animals.

4.11. Adenylyl cyclase (AC) activity

AC activity increased in unstressed WT animals when it was stimulated by forskolin, which was abolished by CL 316243 (Fig. 4.11.1.a). CL 316243 alone had no effect on AC activity in unstressed animals (Fig. 4.11.1.a). In contrast, CL 316243 was able to increase AC activity after one (Fig. 4.11.1.c) and 7 days of cold (Fig. 4.11.1.e). In stressed animals, forskolin also increased the amount of cAMP produced by AC, but CL 316243 did not abolish these effects (Figs. 4.11.1.c, e). In KO animals, forskolin was not able to increase the AC in unstressed animals (Fig. 4.11.1.b), there was only difference between combined treatment with CL 316243 with forskolin and other groups. When stress was applied (after 1 or 7 days) there was marked increase after forskolin treatment (Fig. 4.11.1.d, 1 day, Fig. 4.11.1.f., 7 days). In

contrast to WT animals, CL 316243 had almost no effects on AC activity in KO animals itself (Fig. 4.11.1.b, d, f); it only abolished the effects of forskolin after 7 days of cold.

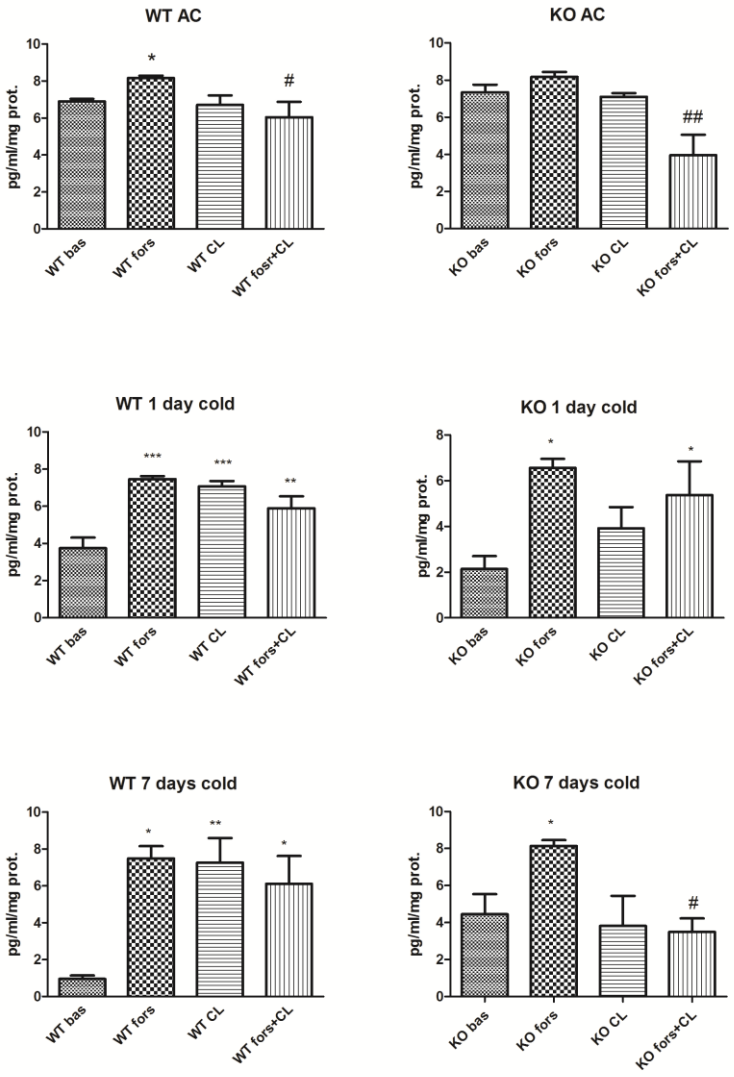

Fig 4.11.1 Changes of adenylyl cyclase activity during exposure to cold in wild type and KO animals.

Abscissa: type of the drug added to the mixture (bas: basal level, i.e., no drug was added, fors: forskolin, CL: CL 316243, fors+CL: forskolin and CL 316243 were applied). Ordinate: the amount of measured cAMP expressed as $pg.ml^{-1}.mg$ of protein A: WT AC: unstressed WT animals, B: KO AC: unstressed KO animals, C: WT 1 day cold, D: KO 1 day cold, E: WT 7 days cold, F: KO 7 days cold.

4.12. Nitric oxide synthase (NOS) activity

BRL 37344 was not able to stimulate NOS activity both in WT animals and KO animals Similarly, the stress had no effect on NOS activity in WT animals (after 1 or 7 days in cold) as well as in KO animals.

4.13. Catecholamine blood concentration

Both adrenaline (A) and noradrenaline (NA) concentrations did not differ between KO and WT animals.

4.14. Gene expression of catecholamine-synthesizing enzymes

There was no difference in the gene expression of tyrosine hydroxylase and dopamine-β-hydroxylase in both WT and KO and in basal state and upon cold stress. There was a slightly higher level of gene expression of phenylethanolamine-N-methyl-transferase in WT animals after 1 and 7 days of cold.

5. Discussion

Our results show that the disruption of M_2-MR gene caused changes in the gene expression of M_3-receptors, β_1-adrenoceptors, and β_2-adrenoceptors together with the presumed decrease in muscarinic receptor binding sites as well as β_1-and β_2-adrenoceptor binding sites (to 52 and 55 %, respectively). The decrease of β_1-adrenoceptor and β_2-adrenoceptor binding sites was caused by changes in the gene expression of the respective gene. In contrast to that, β_3-adrenoceptor binding was increased at the baseline. This phenomenon, i.e. that when one subtype receptor down-regulation occurs and it is followed by down-regulation of an antagonistic receptor as well was demonstrated multiple times (Werry et al., 2003, Myslivecek et al., 2007) and it is fully in accordance with our hypothesis. Since the regulation is region specific (present in the left but not the right ventricle) it is the most likely that heterologous regulation (i.e. local mechanism) is the responsible mechanism for this phenomenon (Werry et al., 2003). This is an important fact suggesting that each ventricle has distinct mechanisms for receptor regulation. Importantly, binding of muscarinic-specific radioligand in M_2KO animals was not completely abolished, confirming the previous finding about another (minor) muscarinic population in the heart (Ito et al., 2009). On the level of gene expression, we were able to detect M_2-, M_3-, and M_4-receptor gene expression in WT animals; M_1- and M_5- receptors were not expressed. This is in a good agreement with some previously published data (Li et al., 2010, Pan et al., 2012), but the difference between species may play a role as well (Myslivecek et al., 2008). Further, we have found a progressive decrease in M_3-gene expression upon cold stress in WT mice but not in M_2KO mice. The expression of M_4-receptors was not altered. M_3-receptors were showed to mediate cardioprotective role of acetylcholine on myocardium; they were showed to ameliorate ischaemia-induced arrhythmias (Wang et al., 2012) and decrease of infarct size and mortality rate employing the M_3/NF-κB/miR-376b-5p/BDNF axis (Pan et al., 2012). This supports the important role of non-M_2-receptors for cardiac function. In our study, we have not found a compensatory up-regulation of M_4-receptors (that together with M_2-receptors inhibit adenylyl cyclase), so this hypothesis was not confirmed.

The receptor binding study on β_3-adrenoceptors showed a baseline increase in M_2KO that was sustained over the period of 7-day stress compared to WT mice, where an increase in β_3-AR appeared only after a rather prolonged stressful stimulus (7-day cold stress). This finding is in a good agreement with previously published studies and with the general paradigm on the function of β_3-adrenoceptors - they are perceived to be a „brake“ that is activated upon excessively increased sympathetic overstimulation causing a negative inotropic effect counteracting the effects of β_1 and β_2 activation (Moens et al., 2010). An application of 7-day cold stress was necessary to provoke an adrenergic response great enough to induce an up-regulation of β_3-AR. Our results show that CL 316243 (10,000 times more selective to β_3-adrenoceptors than to β_1- and β_2-adrenoceptors) was able to abolish the forskolin effects in WT unstressed animals. In contrast to that, CL316243 increases the amount of produced cAMP in cold, which suggests that β_3-adrenoceptors can switch from Gi coupling to Gs coupling. This is the property of β_3-adrenoceptors in the adipose tissue (Hamilton and Doods, 2008). Another possibility is that it can reveal the secondary state of β_3-adrenoceptors as it has been demonstrated earlier (Baker, 2005). Nevertheless, the increase of cAMP production by the β_3-AR activation is unobvious and can be seen in cold stressed WT animals only. Conversely, CL 316243 was not able to increase cAMP levels in KO animals, where β_3-AR are constantly increased. This fact gives evidence about the compensatory role of β_3-AR in the heart when M_2-MR are missing, and suggests that adenylyl cyclase is importantly affected by β_3-adrenoceptor agonist, which suggests that under certain circumstances (cold stress) adenylyl cyclase can be regulated by β_3-AR. This finding does not correlate well with the previous work suggesting that β_3-AR signaling occurs via the activation of NO synthase and subsequent production of NO (Brixius et al., 2004, Amour et al., 2007, Maffei et al., 2007), but it illustrates the complexity of β-AR signaling and well supports the concept of „signalome“ where an individual adrenoceptor associates with multiple signaling pathways in time and spatially specific manner (Park and Palczewski, 2005). Overall, since the main difference found was on the level of receptors, we propose that they serve as a tool to compensate M_2-knockout to preserve cardiac function.

It should be mentioned that there is a mutual connection between heart rate, activity and temperature (both the temperature of animals and ambient temperature). We have found that M_2KO mice have slightly (6%) but significantly increased heart rate (both during light phase and dark phase). In humans, the sinoatrial node is under the dominant influence of parasympathetic nervous system as the resting heart rate is much slower than the intrinsic rate of the sinoatrial node (Brubaker and Kitzman, 2011). On the other hand, it was long believed

that in mice the intrinsic rate of the sinoatrial node is significantly lower than the actual heart rate (Gehrmann et al., 2000), which was interpreted that mice were under a dominant tonus of the sympathetic nervous system. It has been found recently that the ratio between sympathetic and parasympathetic tone depends greatly upon the ambient temperature. At 30°C, WT mice have the heart rate about 350 bpm whereas M_2KO mice 416 bpm (during the light phase) demonstrating that under these conditions the heart rate in mice is (similarly to humans) under dominant tone of parasympathetic nervous system (Swoap et al., 2008). However, when the ambient temperature drops to 21-23°C (normally used in animal facilities), the heart rate rises to cca 600 bpm (light phase) both in WT and M_2KO mice and the difference between groups disappears. Therefore, we have measured ambient temperature very carefully and have showed that in such well controlled environment (24.45 °C light phase/24.21 °C dark phase) it was possible to reveal a slight but significant increase in the heart rate in M_2KO animals, which is fully in accordance with previously published studies (Swoap et al., 2008). On the other hand, some previously published work (Bymaster et al., 2001, LaCroix et al., 2008) reported no difference in the heart rate between WT and M_2KO mice. However, certain factors have biased these observations. Both of these studies were performed on anesthetized animals, the experiments were performed during the day and the ambient temperature was not provided. We believe we were able to record a difference between WT and M_2KO mice because we used telemetric monitoring on wake animals and monitored the ambient temperature very carefully.

The long term telemetric analysis of activity (averaged values and biorhythm analysis) of the animals did not find any difference between WT and M_2KO mice. This finding is in accordance with previously published data (Gomeza et al., 2001). No difference in activity (KO vs. WT) together with lower body temperature in KO animals in relation to unchanged left ventricular function as assessed by echocardiography makes the possibility that the heart rate increase is caused by higher heart performance improbable. This data suggest that M_2-receptors do not affect activity behavior in mice. On the other hand, the long term measurement of the basal temperature of M_2KO mice revealed that is was decreased (without change in biorhythm). This can explain the differences in oxotremorine-induced tremor and hypothermia in M_2KO mice as published previously (Gomeza et al., 1999).

No changes were found in any echocardiographic parameters (related to left ventricular systolic and diastolic function). This observation is in agreement with previously published work (LaCroix et al., 2008) but in contradiction with study when M_2-MR were knocked-out postnatally (a study using M_2-MR coding plasmid DNA-immunized mice) (Gimenez et al.,

2005), which indicates the important differences between pre-natal and postnatal adaptation processes. Preserved systolic and diastolic function is fully in accordance with our hypotheses.

The effects of carbachol in WT animals were expectable. On the other hand, the tachycardia in M_2KO animals was not regularly observed. (Gomeza et al., 1999) referred that incubation of atria derived from M_2KO mice with the carbachol (1μM) had no significant effect on atrial beating frequencies. Similarly, in vivo study with carbachol in M_2KO mice (Bymaster et al., 2001) showed no influence on the heart rate. On the other hand, (Kitazawa et al., 2009) described an increase in inotropy in the atria of M_2KO animals. There are at least two explanations of this phenomenon that originate from our results: (a) the first possibility is that the decrease of β-adrenoceptors (to approximately one half of control values) when heart muscarinic receptors are almost absent in the heart is able to cope with basal conditions of the heart while it is not sufficient to cope with the sustained activation of muscarinic receptors; (b) the second possibility is that the effects of minor muscarinic receptor subtype (as described by (Willmy-Matthes et al., 2003, Wang et al., 2012) and considered as cardiostimulatory) can be revealed whilst it is hidden (in WT animals) by action mediated via major M_2-receptors. According to our knowledge, this is the first observation of cardiostimulatory effects of carbachol *in vivo* in mice lacking the main cardioinhibitory muscarinic receptor subtype. This phenomenon further confirms the role of minor muscarinic receptor subtype in the heart. The third possibility is increased noradrenaline release from sympathetic nerve endings as described by (Trendelenburg et al., 2005) in M_2/M_3KO, but this rather would not be the case as we have not found a difference in blood catecholamines between WT and KO animals.

Upon the application of isoprenaline, we have observed an increase in HR both in WT and KO mice, but the effect on M_2KO animals was attenuated. This observation is in the contradiction with the previously published data (LaCroix et al., 2008) that reported a significantly increased heart rate upon isoprenaline application in M_2KO mice. The reason why we have observed opposite results is not clear; however, in our study all the measurements were performed by telemetry without using sedation, which we believe helped to obtain more reliable and unbiased results. The lesser extent of tachycardia in KO animals treated with isoprenaline can have the nature in having a decreased number of β-AR (52% for β_1-AR and 55 % for β_2-AR). On the other hand, the effects of β-AR agonist persisted in KO animals, suggesting the compensatory role of receptor changes in heart physiology.

Atropine application increased the heart rate in WT animals but did not have this effect in M_2KO, confirming the absence of main cardioinhibitory receptors (M_2) in the heart. It was reported previously that atropine had no effect in WT animals (LaCroix et al., 2008), which is in contradiction with our results. Again, we believe that the measurements performed by telemetry without using sedation helped us to obtain more reliable and unbiased results.

Propranolol decreased the heart rate both in WT and M_2KO animals; however, the drop in M_2KO animals was attenuated (a decrease to 48% in WT but only to 70% in M_2KO). This supports the hypothesis mentioned earlier that the decrease in β-adrenoceptors could be responsible for the diminished effects of β-adrenoceptor drugs in M_2KO animals. Once again, these data also support the hypothesis about the compensatory role of receptors in maintaining stable and physiological cardiac output.

6. Conclusion

In cardiac left ventricles, the disruption of M_2-receptor gene caused a compensatory decrease of cardiostimulatory β_1-adrenoceptors and β_2-adrenoceptors with corresponding down-regulation of the gene expression, M_3-receptors were down-regulated as well. Missing M_2-receptors were functionally replaced by the main cardioinhibitory β_3-adrenoceptors that were up-regulated, not by cardioinhibitory M_4-receptors. β_3-adrenoceptors were found to signal through adenylyl cyclase instead of NO synthase. All these changes were found in the left ventricle only, so heterologous regulation is likely to be the responsible mechanism. Slightly higher basal heart rate and lower basal body temperature of M_2KO animals underscore the importance of telemetric measurement to avoid biased recordings. Despite all the changes found the overall cardiac function was unaltered.

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1. publikace *in extenso*, které jsou podkladem dizertace

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