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Research Article

Muscarinic M_3 receptor subtype gene expression in the human heart

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Abstract. The heart is an important target organ for cholinergic function. In this study, muscarinic receptor subtype(s) in the human heart were determined using reverse transcription-polymerase chain reaction. Our results demonstrated muscarinic receptor M_2 and M_3 subtype RNA in left/right atria/ventricles of donor hearts. Receptor autoradiography analysis using selective muscarinic ligands indicated an absence of M_1 receptor subtype in the human heart. The level of muscarinic receptor binding in atria was two to three times greater than in ventricles. Our results suggest that muscarinic receptors in the human heart are of the M_2 and M_3 subtypes. This is the first report of M_3 receptors in the human myocardium.

Key words. Muscarinic receptor; subtypes; human; heart failure; ventricle; atrium; mRNA; protein.

Muscarinic cholinergic receptors play an important role in the regulation of cardiac activities, such as the rate and force of contraction. To date, five muscarinic receptor subtypes (M_1–M_5) have been reported. These are encoded by five different intronless genes [1–8]. Several studies have addressed the classification of muscarinic receptor subtypes in animal and human heart by means of radioligand-binding techniques. Fields et al. [9] demonstrated the presence of a single population of myocardial muscarinic receptors in the hearts of guinea pig, rabbit, and rat. Doods et al. [10], using M_1, M_2, and M_3 subtype-selective muscarinic receptor antagonists, reported that in rat atria and ventricles, the muscarinic receptors mediating negative chrono- and/or inotropic effects are exclusively of the M_2 subtype. Similarly, in studies of the human heart, the presence of only the M_2 muscarinic receptor subtype has been shown by radioligand binding [11, 12] and functional experiments (negative inotropic effect of carbachol on isolated electrically driven right atrial or left papillary muscle preparations) [12]. Moreover, in receptor-binding studies, the atrial regions of rabbit, rat [9], chick [13], and human [12] hearts were shown to be more densely endowed with M_2 receptors than the ventricular myocardium. The density of receptor binding in these species was similar in right and left atria and, likewise, for right and left ventricles. Presynaptic M_1 muscarinic receptor expression was suggested to occur in the sinus node of human heart [14]. However, the results of that study indicated negative chronotropic effects of pirenzepine and atropine only in the native atrial cuffs but not in the transplanted donor hearts, i.e., only when innervation was intact. The authors concluded that the final experimental proof for the existence of such a presynaptic M_1 subtype in the human atrium is lacking. Using oligonucleotide probes specific to M_1, M_2, M_3,

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and M_4 muscarinic receptor mRNAs, Hoover et al. [15] demonstrated the presence of the M_2 isoform in the rat myocardium and M_1, M_2, and M_3 isoforms in the rat intrinsic cardiac ganglia. Similarly, by means of Northern blot analysis with M_1–M_3 specific probes, only the M_2 subtype was detected in porcine heart [16]. However, when the reverse transcription-polymerase chain reaction (RT-PCR) was performed, not only M_2 but also M_1 subtype was found in adult rat [17] and guinea pig [18] ventricular myocytes. The presence of M_1 and M_2, but not M_3, muscarinic receptor proteins on the surface of rat ventricular myocytes was confirmed by immunocytochemical analysis [17]. Recently Shi et al. [19] identified M_2, M_3, and M_4 subtypes in canine atrial myocytes. They confirmed their ligand-binding findings of M_2–M_4 in dog atrial myocytes by cloning cDNA fragments and detecting mRNAs corresponding to M_2–M_4 subtypes. Moreover, functional studies of muscarinic receptor subtypes suggested that M_3 exists in canine and guinea pig atrial myocytes and it coupled to K^+ channels [19–21].

It is difficult to identify pharmacologically M_{1-5} receptor subtypes due to the lack of highly selective ligands to all five subtypes. Therefore, analysis at the RNA level is useful for this purpose. The aims of the present study were to investigate, by means of RT-PCR, which muscarinic receptor subtype(s) exist in different regions of human heart and to find possible alterations in subtype gene expression in patients with dilated cardiomyopathy (DCM). RT-PCR was performed with primers specific to all known (M_{1-5}) muscarinic receptor subtypes.

Materials and methods

Normal hearts. Myocardial specimens were obtained from brain-dead kidney and liver donors [n = 8, mean age 24 (14–42) years old]. The heart weight range was 240–340 g. According to patient records, none of the organ donors had any known disease or had used any drugs. Myocardial specimens were obtained within 20 h of the diagnosis of brain death and the specimens were taken within 15–30 min after the heart had stopped beating. The regions analyzed were from the subendocardium of the free wall of the left ventricle, from the free wall of the right ventricles, and from the atria. Each sample was immediately frozen in liquid nitrogen and stored at −70 °C until analyzed.

Diseased hearts. Biopsies from hearts with DCM were obtained from patients with biventricular end-stage heart failure, undergoing orthoptic heart transplantation (n = 6). The age of the patients was 26–56 years and New York Heart Association functional group was III–IV. Pretransplant hemodynamic indices were markedly abnormal with an ejection fraction of $23 \pm 5\%$ and a pulmonary vascular resistance of 2.0 ± 0.8 mm Hg l^{-1}. Endomyocardial biopsy was used for pretransplant testing to exclude specific causes of cardiomyopathy, especially hemochromatosis, thyreotoxicosis, and connective tissue disease. Pharmacological therapy included digoxin, diuretics, and nitrates. Biopsies were taken from the subendocardium of the free wall of the left and from the free wall of the right ventricles.

The morphology of normal and diseased cardiac tissues was analysed to exclude regions containing vascular vessels.

Ethics. All work was performed in accordance with the Declaration of Helsinki. The study was approved by local ethical committees.

RNA preparation and RT-PCR. Total cellular RNA was extracted from cardiac tissue of donor hearts and left and right ventricles of DCM patients (5–10 mg of tissue by the method of Chomczynski and Sacchi [22]). The extracts were treated with 3 U of RNase-free DNase I (Boehringer Mannheim) at 37 °C for 30 min to eliminate DNA contamination. RNA samples were extracted with phenol-chloroform and precipitated with ethanol. The RT reaction to make cDNA was performed using deoxynucleotides (Tamro), RNasin (Boehringer Mannheim), random hexanucleotide primers (Boehringer Mannheim) and Superscript reverse transcriptase (Gibco BRL) as follows. The mixture of RNA and random hexamer primers was incubated for 10 min at 70 °C, cooled on ice, incubated for 2 min at 37 °C with a reaction mixture containing 5 × buffer, DTT, H_2O, deoxynucleotides and RNasin, and after addition of Superscript reverse transcriptase, incubated for 60 min at 37 °C, 10 min at 42 °C and 10 min at 95 °C. PCR was carried out with M_1–M_5 subtype-specific primers for 40 cycles (94 °C for 40 s, 55 °C (M_1, M_2, M_3 primers) or 60 °C (M_4, M_5 primers) for 40 s and 72 °C for 1 min with a final extension for 7 min at 72 °C. DMSO (2.5%) was included in the PCR solution for amplification of the M_4 subtype. The sequences of the primers used and their positions and lengths are decribed in Hellström-Lindahl and Nordberg [23]. The products of RT-PCR were analyzed by 1% agarose gel electrophoresis. Sizes of expected PCR products were 573 bp (M_1), 469 bp (M_2), 434 bp (M_3), 592 bp (M_4), and 451 bp (M_5). RNA samples that had not been transcribed to obtain cDNA were used as negative controls. Negative and positive controls were included in the PCR runs. The identity of PCR products was confirmed by restriction analysis with the following enzymes (Promega): *Pst*I for M_1, M_3, and M_4, *Bam*HI for M_2 and *Dde*I for M_5. The sizes of restriction fragments were analyzed by the DNA Strider program.

Receptor autoradiography. Frozen tissues from donor human hearts and from the left ventricles of patients with DCM were mounted for sectioning in a Leitz cryostat. Sections of 10 μm were cut and thaw-mounted onto chrome/gelatin-coated microscope slides for receptor autoradiography. Slide-mounted sections were preincubated in cold 50 mM Na-K phosphate buffer (pH 7.4) for 30 min. The sections were then incubated with the following radiolabelled ligands: ^{125}I-muscarinic toxin-7 [^{125}I-MT-7, 2 nM, M_1 selective, (buffer contained 1% BSA (w/v)] or ^{3}H-N-methyl-scopolamine ([^{3}H]NMS, nonselective), 0.5 nM, in the above buffer for 90 min at 25 °C. Nonspecific binding was defined by inhibition in the presence of atropine (10^{-4} M). At the end of the incubation period, the sections were rinsed in the buffer to terminate the assay and dipped in distilled water to remove buffer salts. Subsequently, sections were dried under a stream of cold air and tightly juxtaposed to tritium-sensitive films (Hyper film ^{3}H, Amersham, UK) in X-ray cassettes for 30 days (for ^{125}I-MT-7, 3 days) at − 20 °C. Following exposure, the films were removed and developed. For calibration, radioactive standards were exposed and developed with the sections to permit conversion of optical densities to molar quantities of receptor-bound radioligand. The quantitative analysis of autoradiographs was performed using a NIH image 1.55.2 VDM-PM analyzer for densitometric analysis of the film sheets. Quantification of each radioligand binding to heart tissues was performed in six sections from six human hearts. Specific binding was obtained by subtracting nonspecific binding from total binding. The specific binding was expressed as femtomoles per milligram tissue wet weight.

The concentrations of ^{125}I-MT-7 and [^{3}H]NMS were calculated as follows. The MT-7 was iodinated by the chloramine T method [24]. Briefly, 10 μg of MT-7 was iodinated using ^{125}I. The bound was separated from the free ^{125}I by gel filtration. Aliquots were taken from all the eluents for scintillation counting to determine the ^{125}I-MT-7 from the free ^{125}I peak. The specific activity of ^{125}I-MT-7 was then calculated from the counts per minute obtained. The concentration of ^{125}I-MT-7 was then calculated by standard methods. In the case of [^{3}H]MS, the specific activity (Ci/mmol) was given by the supplier and hence the concentration was calculated directly by standard procedures. Disintegrations per minute (DPM) was determined using a beta scintillation counter in 2 μl of the stock solution. From the DPM and specific activity, the molar concentration was obtained. The amount required for the experiment was then taken from the stock and diluted to the desired concentration.

Results

Identification of muscarinic receptor subtypes in human heart by RT-PCR. Total RNA was extracted from right and left atria, right and left ventricles of donors hearts, as well as from the left and right ventricles of patients with DCM. The expression of genes encoding the M_1–M_5 muscarinic acetylcholine receptor subtypes in human heart was analyzed using RT-PCR. Each analysis was performed three times. To confirm that PCR products were amplified from cDNA and not from genomic DNA, PCR was also performed with isolated RNA before cDNA was synthesized by reverse transcriptase and was used as negative control (data not shown). Positive controls of PCR for all primers (M_1–M_5) were performed on RT-PCR samples obtained from rabbit and human fibroblasts and human blastocytes (fig. 1). Only M_2 and M_3 subtypes were found in the donor hearts (fig. 1). Both M_2 and M_3 were present in all cardiac compartments in all the eight donor hearts. The size of M_2 (469 bp) and M_3 (434 bp) PCR products corresponded to those theoretically expected. Similar to donor hearts, the M_2 gene was expressed in right and left ventricles of all DCM patients (fig. 1). However, the M_3 subtype was found in only 2/3 of patients with DCM. Messenger RNAs corresponding to M_1, M_4, and M_5 subtypes were not detected in donor or failing hearts.

Muscarinic receptor autoradiography study. The presence of muscarinic receptors at the protein level was analyzed by in vitro quantitative receptor autoradiography using the M_1-selective toxin ^{125}I-MT-7 or the nonselective [^{3}H]NMS. Receptor autoradiography analysis showed the absence of M_1 receptor subtype in the heart (results not shown). The binding of [^{3}H]NMS revealed an uneven distribution of muscarinic receptors in the different compartments of donor human hearts: atria had two- to three-fold more receptors than ventricles (fig. 2). The density of receptor binding was similar in right and left regions of atrium and likewise in right and left ventricles.

Discussion

The purpose of this work was to clarify which subtypes of the muscarinic receptor are expressed in different regions of donor human heart and in left and right ventricles of patients with DCM, using RT-PCR. Furthermore, muscarinic receptor levels in ventricles and atria of donor hearts were analyzed by receptor autoradiography.

In most previous reports, the presence of muscarinic receptor subtypes in animal and human hearts was studied by radioligand-binding techniques. However, this technique is limited due to lack of selective ligands for all the muscarinic receptor subtypes.

In this study, the presence of muscarinic receptors in the human heart was investigated by RT-PCR with primers specific to all five muscarinic receptor subtypes. We found that only M_2 and M_3 subtypes are present in atria and ventricles of human donor heart. This is the first report of the presence of the M_3 muscarinic receptor subtype in the human heart. Receptor autoradiography analysis, using an M_1-selective muscarinic ligand, showed the absence of M_1 receptor subtype in the human heart. These findings are in line with the RT-PCR results. The lack of gene expression in human hearts of M_1, M_4, and M_5 muscarinic receptor subtypes, as demonstrated by RT-PCR, suggests that the [^{3}H]NMS binding observed using receptor autoradiography may be to M_2 and M_3 subtypes. Using quantitative receptor autoradiographic analysis, we have also demonstrated that the level of muscarinic receptors in human hearts is much higher in the atria than in the ventricles. Our data are in agreement with previous studies of muscarinic receptor density in the atrial and ventricular regions of rabbit, rat [9], chick [13], and human [12] hearts. However, our results contrast with previous reports where by means of radioligand binding, only the M_2 subtype was shown in human atrial and ventricular myocardium [11, 12]. This discrepancy may partly be explained by differences in the methods used. Similar contradictions are observed for example in studies of rat hearts. Using radioligand-binding techniques, a single population of muscarinic receptor [9] or only M_2 subtype [10] were shown. The latter results agree with those of an in situ hybridization study on rat myocardium mRNAs with M_1- to M_4-specific probes, where the M_2 subtype was exclusively found [15]. How-

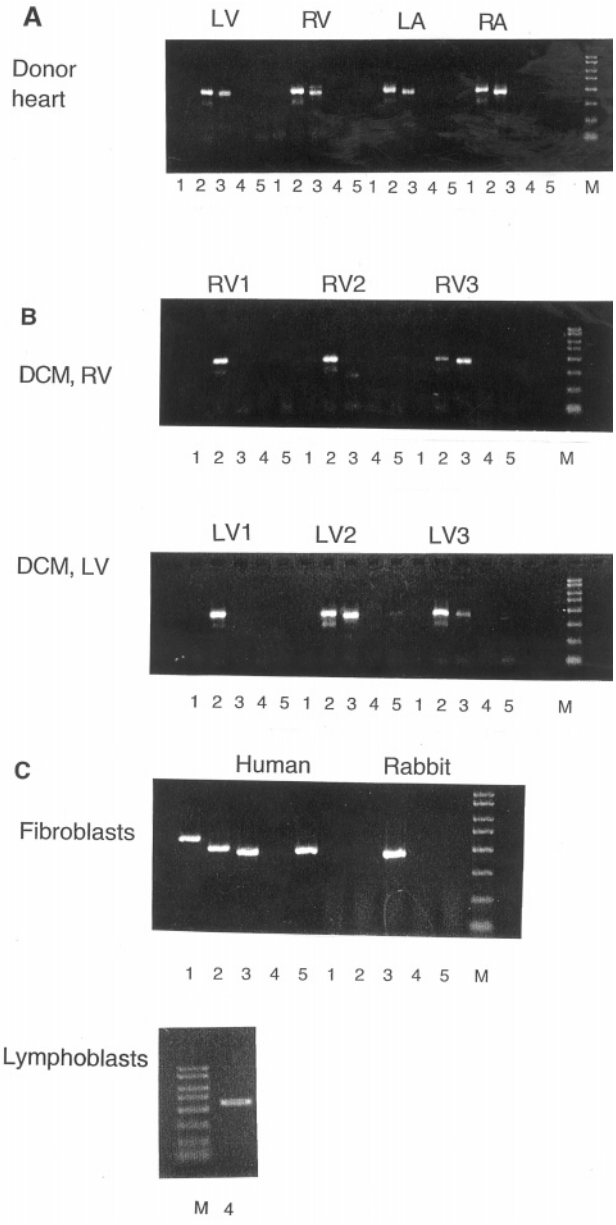

Figure 1. RT-PCR analysis of M_1–M_5 muscarinic receptor subtype gene expression in human heart. Representative 1% agarose gel electrophoresis shows the presence of M_1–M_5 muscarinic receptor subtype mRNA in donor heart (*A*) and in ventricles of DCM patients (*B*). Positive controls for M_1–M_5 primers (*C*): RT-PCR was performed on mRNA samples obtained from human and rabbit fibroblasts and human lymphoblasts. Lane 1, M_1; lane 2, M_2; lane 3, M_3; lane 4, M_4; lane 5, M_5. LV, left ventricle; RV, right ventricle; LA, left atria; RA, right atria. The DNA marker (lane M) is represented by bands of 2000, 1500, 1000, 750, 500, 300, 150, and 50 bp.

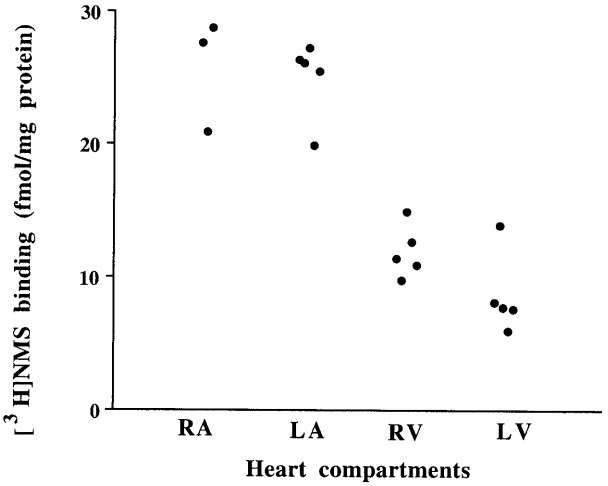

Figure 2. Autoradiography analysis of muscarinic receptor binding in human heart. The level of muscarinic receptors was measured in different compartments of donor human heart using [^{3}H]NMS. LV, left ventricle; RV, right ventricle; LA, left atria; RA, right atria.

ever, in other reports, radioligand-binding analysis revealed evidence for an additional subtype of rat myocardial muscarinic receptor [25, 26]. This additional isoform was defined more precisely by RT-PCR where not only the M_2 but also an M_1 subtype was demonstrated in adult rat ventricular myocytes [17].

It seems that expression of myocardial muscarinic receptor subtypes may be species dependent. Thus rat [17] ventricular myocytes have been shown to contain M_1 and M_2 subtypes, while chick appears to contain three subtypes: receptor: M_2, M_3, and M_4 [27–29]. If there is a difference in MR gene expression in atrial and ventricle regions remains to be clarified. For example, studies with guinea pigs showed the presence of M_1 and M_2 in ventricular myocytes [18], while the M_3 subtype was identified in atrial myocytes [20]. Our findings revealed the presence of M_2 and M_3 subtypes in left and right atrial and ventricular regions of the human heart.

In our study of patients with DCM, the M_2 isoform was demonstrated in all samples from left and right ventricles, while the M_3 subtype was shown in only 2/3 of them. Thus DCM may influence expression of the gene encoding the M_3 muscarinic receptor.

In conclusion, our study indicates (i) the gene expression of only M_2 and M_3 muscarinic receptor subtypes in the human heart, (ii) that the level of muscarinic receptor binding was two to three times higher in the atria than in the ventricles of human heart. Additional studies are required to determine the physiological role of the novel human M_3 cardiac receptor subtype in healthy and disease states.

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