

5-Hydroxytryptamine synthesis in HL-1 cells and neonatal rat cardiocytes

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Abstract

Some reports showed that serotonergic system might have existed and that 5-hydroxytryptamine (5-HT) was detected in the hamster heart. The source of 5-HT in the heart, however, remains to be fully elucidated. So the present study was designed to define serotonergic system and to clarify which cell could produce 5-HT in the heart. As a result, 5-HT was detected in homogenates of HL-1 cardiomyocytes by high performance liquid chromatography with fluorescence detection, but not in those of neonatal rat non-cardiomyocytes (NMCs). And TPH and AADC mRNAs were expressed in HL-1 cardiomyocytes and neonatal rat cardiomyocytes (MCs), not in NMCs. mRNAs of 5-HT_{2A} receptor were detected in both MCs and NMCs, and those of 5-HT_{2B} receptor in NMCs. These findings definitively demonstrate that 5-HT is secreted from the myocytes of the heart and strongly implied that 5-HT might play a certain role in cardiac physiology.

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Several studies reported that 5-HT was demonstrated in the hamster heart and involved in cardiac pathophysiology [1–4]. We previously showed that sarpgrelate, a specific inhibitor of 5-HT_{2A} receptor, attenuated [³H]leucine uptake into neonatal Wistar rat ventricular cardiac myocytes (MCs) but failed to show the evidence of 5-HT secretion from MCs [5]. Recent studies revealed that the tryptophan hydroxylase (TPH) mRNA is expressed in the heart [6] and that immunostaining of 5-HT is positive in fetal heart [7]. These results indicate

that 5-HT may be produced both in fetal MCs and in adult MCs. But previous studies did not clarify the precise source of 5-HT in postnatal cardiocytes. So the present study was designed to define the evidence of 5-HT synthesis in both HL-1 cardiomyocytes and neonatal ventricular cardiac myocytes (MCs) and non-myocytes (NMCs).

Materials and methods

All experiments were performed in accordance with the *Guidelines on Animal Experimentation of Jikei University*.

Preparation of HL-1 cardiomyocytes. HL-1 cardiac myocytes (passages 72–73) [8] were plated in Ø 10 cm culture dishes pre-coated

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with 0.02% Bacto gelatin (Becton–Dickinson, Sparks, MD) and 5 μ g/ml fibronectin (Sigma–Aldrich, St. Louis, MO), and cultured until cells reached confluence in Claycomb medium (JRH Biosciences, Lenexa, KS) supplemented with 10% fetal bovine serum (FBS), 0.1 mM norepinephrine, and antibiotics (maintenance medium). Cardiomyocytes were maintained until they reached confluence. When HL-1 cardiomyocytes reached confluence, cells were removed by trypsinization and passed into another \varnothing 10 cm culture dishes coated with gelatin/fibronectin.

Preparation of rat neonatal MCs and NMCs. MCs and NMCs, which consisted mainly of cardiac fibroblasts, were prepared from cardiac ventricles of 2–4-day-old neonatal Wistar rats by the Percoll gradient and adhesion methods as previously described [5]. MCs were suspended in Iscove's modified Dulbecco's medium (pH 7.4, IMDM, Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS, Sigma–Aldrich), non-essential amino acid solution (1 ml/L, Invitrogen), and antibiotics. Then, MCs were plated in \varnothing 10 cm culture dishes at a density of 1.8×10^6 cells/dish. MCs were also plated in chamber slide (Iwaki Glass, Funabashi, Japan) for certification of the purity and were cultivated overnight. After fixation with 10% neutral formalin, cells were stained with anti-sarcomeric actin antibody (ZMSA-5 (180177), Zymed Laboratory, South San Francisco, CA) and HISTOSTAIN-SP Kit (Zymed Laboratory). NMCs were suspended in IMDM containing 10% FBS and antibiotics, and plated in \varnothing 10 cm culture dishes. The dishes were washed with Hanks' buffered salt solution (Sigma–Aldrich) four times after 30-min incubation to remove endothelial cells, and NMCs were incubated in DMEM containing 10% FBS and antibiotics. When NMCs reached subconfluence, they were removed by trypsinization and again cultured in \varnothing 10 cm culture dishes with DMEM containing 10% FBS and antibiotics (the 1st passage).

High performance liquid chromatography for 5-HT. When HL-1 cardiomyocytes and NMCs (the 2nd passage) reached confluence in \varnothing 10 cm culture dishes, cells were collected by trypsinization and washed twice with phosphate-buffered saline (PBS). Then, cells were transferred into 1.5 ml centrifugation tubes and centrifuged at 10,000g for 5 min at 4 °C. After discarding of PBS, cell pellets were homogenized in 1 ml of 0.5 M perchloric acid (Kanto Chemical, Tokyo, Japan) containing 0.02% ascorbic acid (Sigma–Aldrich) followed by centrifugation at 10,000g for 30 min at 4 °C. The supernatant is removed and stored at –80 °C until analysis. One hundred microliters of samples for 5-HT analysis was injected into post column high performance liquid chromatography (HPLC) system (LC10-A, Shimadzu, Kyoto, Japan) including guard column (TSK Guardgel ODS-80TM, 3.2×15 mm, Tosoh, Tokyo, Japan) with fluorescence detection (excitation: 345 nm, emission: 480 nm). Mobile phase consisted of acetonitrile–10 mM acetate buffer (pH 4.7, 1.0 ml/min) and mixed acetonitrile–25 mM borate buffer containing (pH 10.0, 0.5 ml/min) 20 mM benzylamide and 3 mM potassium ferricyanide ($K_3Fe(CN)_6$) (purchased from Nakalai Tesque, Kyoto, Japan) after sample injection [9]. Data were analyzed by computer

software, CLASS-VP (Shimadzu). Genomic DNA from HL-1 cardiomyocytes and NMCs in each culture to normalize 5-HT production was harvested by lysis in a buffer containing 10 mM Tris–HCl (pH 8.0), 10 mM ethylenediaminetetraacetic acid– Na_2 (pH 8.0), and sodium lauryl sulfate (0.5%) (purchased from Nakalai Tesque), and subsequent treatment with proteinase K (100 μ g/ml, Invitrogen) and RNase A (100 μ g/ml, Sigma–Aldrich). Then, after phenol/chloroform extraction and ethanol precipitation, genomic DNA was quantified by measurement of absorbance at 260 nm. Obtained data on 5-HT were normalized by the amount of genomic DNA in each culture and represented as pmol/ μ g DNA.

Total RNA extraction from HL-1 cardiomyocytes, rat MCs and NMCs, and cDNA synthesis. Rat MCs were cultivated in a \varnothing 10-cm dish at a density of 1.8×10^6 cells/dish for 30–40 h. HL-1 cardiomyocytes and rat NMCs (the 2nd passage) were also cultured in \varnothing 10 cm culture dishes until confluence. Then, cells were washed twice with phosphate-buffered saline and homogenized with 1 ml/dish TRIzol (Invitrogen). Total RNA was extracted by the acid guanidinium–phenol–chloroform method and treated with DNase I (TaKaRa Bio, Otsu, Japan). Five micrograms of total RNA of HL-1 cardiomyocytes and approximately 1 μ g of total RNA of neonatal rat MCs and NMCs were applied to synthesize cDNA with SuperScript II 1st Strand DNA Synthesis Kit (Invitrogen).

Reverse transcription-polymerase chain reaction for 5-HT₂ receptors, TPH, and aromatic L-amino acid decarboxylase mRNA. Reverse transcription-polymerase chain reaction (RT-PCR) for 5-HT₂ receptors, TPH, and amino acid decarboxylase (AADC) was performed using the primers shown in Table 1. Primers for rat 5-HT₂ receptors, rat TPH, mouse AADC, and mouse TPH were synthesized according to previous reports [4,10–12] and primers for rat AADC were synthesized according to GenBank Accession No. NM012545 (designed by primer 3 web site, http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) (Table 1, all primers were synthesized by Sigma Genosys Japan, Ishikari, Japan). PCR was performed using Bioneer AccuPower PCR PreMix (Bioneer, Daejeon, Korea), TaKaRa Ex Taq Hot Start version, and TaKaRa Taq Hot Start version (TaKaRa Bio) under conditions of PCR cycle consisted of denaturing at 94 °C for 1 min, annealing primers to cDNA at each annealing temperature for 1 min, and extension at 72 °C for 2 min (β -actin; 25 cycles, rat TPH; 38 cycles, and others; 35 cycles). Annealing temperatures are as follows; β -actin: 54.4 °C, rat TPH; 64.0 °C, mouse TPH; 60.0 °C, rat AADC; 61.3 °C, mouse AADC; 60 °C, 5-HT_{2A} receptor; 59.2 °C, 5-HT_{2B} receptor; 63.8 °C, and 5-HT_{2C} receptor; 61.0 °C. These PCR conditions for rat primers were certified with cDNA from neonatal Wistar rat brain and, for mouse primers, BD QUICK-Clone cDNA (cDNA clone from mouse brain, lot #3120504, BD Biosciences Clontech, Palo Alto, CA, USA) (data not shown). Finally, PCR using total RNA extract solution from whole heart, MCs, and NMCs, instead of cDNA, which was not reverse-transcribed, was also performed with β -actin primers (25 cycles, 94 °C for 1 min, 54.4 °C for 1 min, and 72 °C for 2 min). PCR products were separated in 2% agarose gel. After gels were stained by

Table 1
PCR primers

	Forward primer	Reverse primer	Estimated amplicon size (bp)
5-HT _{2A} receptor	CATCCTGTATGGGTACCGGT	AAAGACCTTCGAATCATCCTG	265
5-HT _{2B} receptor	AGGCTACATGGCCCCTCCACT	AAAGACCTTCGAATCATCCTG	222
5-HT _{2C} receptor	TATCCCTGTGATTGGACTGAG	GTTGATAGCCTTGCATGGTGC	350
Mouse TPH	TGATGGTTTCCAGTGCATATCC	CGTGGCACGTGAACCTATATTTC	241
Mouse AADC	AGCATGCACAGAGCTGGAGAC	AAGAAAGGAATCAGGCCAGC	386
Rat TPH	CATTCTCAGAAAGGGGGAGAGTGACT	AGCTGATCGGGCGACTCCACAGAGA	254
Rat AADC	ATCTTCTGAATGGCGTGGAG	TTGCCAGTGCCTGTAGTCAG	194 (932–1125)
β -Actin	GACTACCTCATGAAGATCCT	CCACATCTGCTGGAAGGTGG	510

10 $\mu\text{g/ml}$ ethidium bromide, the intensity of ethidium bromide was then detected by ultraviolet transilluminator.

Results

Typical fluorescent response of standard 5-HT (1 μM) with benzylamine and potassium ferricyanide is shown in Fig. 1A. Fluorescent responses of 5-HT with potassium ferricyanide were detected in the extracts from HL-1 cardiomyocytes (64.6 ± 60.9 pmol/ μg DNA, $n = 4$, Fig. 1B), while not detected in the extracts in rat NMCs (Fig. 1C).

Anti-sarcomeric actin-positive cells in neonatal rat MCs were approximately 97% of obtained cells and contamination of genomic DNA was ruled out by PCR performed with total RNA extracts and β -actin primers before cDNA synthesis (data not shown). Mouse TPH and AADC mRNAs were expressed in HL-1 cardiomyocytes (Fig. 2A), and rat TPH and AADC mRNAs were expressed in rat MCs, while both TPH and AADC mRNAs were not detectable in rat NMCs (Fig. 2B). In addition to expression of TPH and AADC mRNA, 5-HT_{2A} receptor mRNA was detected in both rat MCs and NMCs. 5-HT_{2B} receptor mRNA was detected in both neonatal rat MCs and NMCs at 35 PCR cycles but was dominantly expressed in NMCs. Expression of

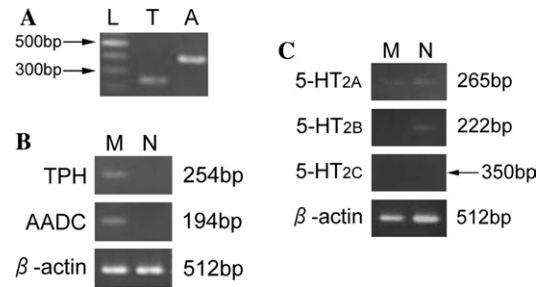


Fig. 2. Expression of TPH and AADC mRNAs in HL-1 cardiomyocytes (A), neonatal rat cardiocytes (B), and 5-HT type 2 receptor mRNAs in neonatal cardiocytes (C). L, 100 bp DNA ladder marker; T, TPH 1; A, AADC; M, neonatal rat MCs; and N, neonatal rat NMCs.

5-HT_{2C} receptor was not detected in both neonatal rat MCs and NMCs (Fig. 2C).

Discussion

It has been reported that 5-HT is involved in cardiac contraction [1,2] and development of fetal heart [4,7]. And it is also demonstrated that amount of 5-HT is decreased in diseased heart compared with normal heart in hamster [3] and disruption of 5-HT synthesis system by mutation of TPH 1 gene (*tph1*^{-/-} mice) displays cardiac abnormalities without structural defects [4], indicating

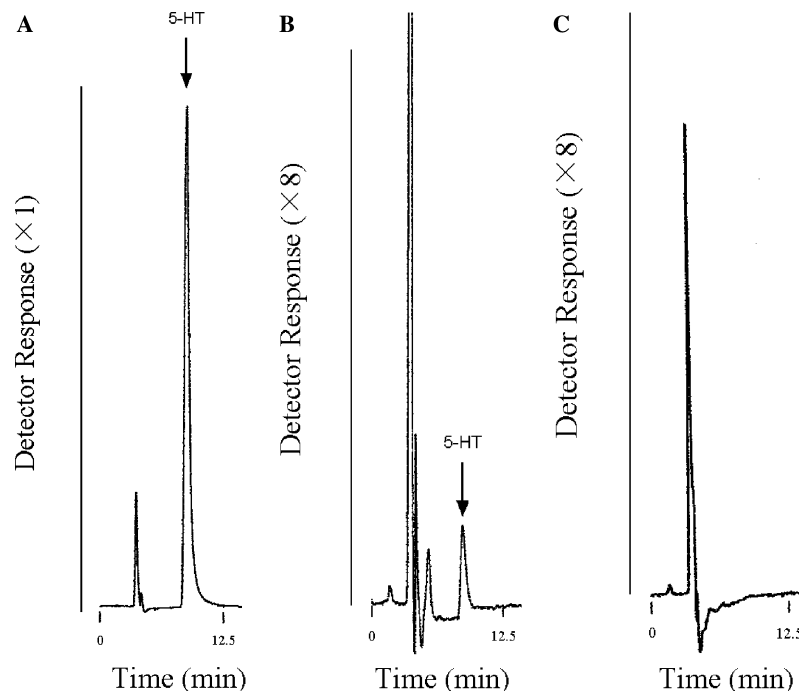


Fig. 1. Representative responses of HPLC detector to 5-HT control (1 μM) and extracts from HL-1 cardiomyocytes and neonatal rat NMCs. (A) Detector response to 5-HT (1 μM , control). (B) Typical detector response to the sample extracted from HL-1 cardiomyocytes. (C) Typical detector response to the sample extracted from cultured rat neonatal NMCs (indicated detector responses in (B,C) are 8-fold of control). No significant responses were detected after 12.5 min in any samples.

that 5-HT may play a crucial role on cardiac development in fetal stage and cardiac pathophysiology in adult heart. Recently, Slominski et al. [6] and we reported that TPH mRNA is expressed in hamster heart and addition of sarpogrelate, a specific 5-HT_{2A} receptor antagonist, by itself results in decrease in incorporation of [³H]leucine into MCs [5]. In addition, a more recent study showed that 5-HT immunoreactivity is detected in fetal heart [7]. In the present study, we used HL-1 cardiomyocytes, which is the atrial cardiomyocyte cell line, instead of ventricular MCs, because it is difficult to obtain enough number of neonatal MCs to measure 5-HT by HPLC with fluorescent detection. But we define that HL-1 cardiomyocytes, a newly developed atrial cardiac myocyte cell line which shows a similar gene expression pattern like adult cardiomyocytes [8], could produce 5-HT and might have 5-HT synthesizing enzymes, while 5-HT was not detected in the extracts from neonatal rat NMCs, which mainly consisted of cardiac fibroblasts (Figs. 1B, 1C, and 2A). The PCR examinations on the expression of 5-HT synthesizing enzyme mRNAs also indicate that 5-HT might be synthesized in neonatal rat ventricular MCs as well as HL-1 cardiomyocytes, but not in rat NMCs. And expression of 5-HT type 2 receptor mRNAs also indicates that 5-HT acts on the heart in autocrine and paracrine fashions (Fig. 2C). Together with these findings, the present study definitively demonstrates that 5-HT is secreted from the myocytes of the heart and strongly implied that 5-HT might play a certain role on cardiac physiology.

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