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Functional Constituents of a Local Serotonergic System, Intrinsic to the Human Coronary Artery Smooth Muscle Cells

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Abstract

Human coronary artery smooth muscle cells (HCASMCs) play an important role in the pathogenesis of coronary atherosclerosis and coronary artery diseases (CAD). Serotonin is a mediator known to produce vascular smooth muscle cell (VSMC) mitogenesis and contribute to coronary atherosclerosis. We hypothesize that the human coronary artery smooth muscle cell possesses certain functional constituents of the serotonergic system such as: tryptophan hydroxylase and serotonin transporter. Our aim was to examine the presence of functional tryptophan hydroxylase-1 (TPH1) and serotonin transporter (SERT) in HCASMCs. The mRNA transcripts by qPCR and protein expression by Western blot of TPH1 and SERT were examined. The specificity and accuracy of the primers were verified using DNA gel electrophoresis and sequencing of qPCR products. The functionality of SERT was examined using a fluorescence dye-based serotonin transporter assay. The enzymatic activity of TPH was evaluated using UPLC. The HCASMCs expressed both mRNA transcripts and protein of SERT and TPH. The qPCR showed a single melt curve peak for both transcripts and in sequence analysis the amplicons were aligned with the respective genes. SERT and TPH enzymatic activity was present in the HCASMCs. Taken together, both TPH and SERT are functionally expressed in HCASMCs. These findings are

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Author Contributions

Kannan Baskar: Conception, design, execution of the experiments, analysis and interpretation of data and writing of the initial draft of the manuscript.

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Vithyalakshmi Selvaraj: Co-mentored and co-supervised Kannan Baskar, involved in the analysis and interpretation of the data.

Devendra K. Agrawal: Co-mentored and co-supervised Kannan Baskar and Swastika Sur, involved in the discussion on developing the project, analysis and interpretation of the data, and critically evaluated and edited the manuscript after each author provided the comments, finalized the manuscript for submission to the journal.

Conflict of Interest

The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

novel and represent an initial step in examining the clinical relevance of the serotonergic system in HCASMCs and its role in the pathogenesis of coronary atherosclerosis and CAD.

Keywords

Atherosclerosis; Coronary artery disease; Serotonin transporter; Tryptophan hydroxylase; Vascular smooth muscle cell

Introduction

Serotonin (5HT) is synthesized from L-tryptophan by the enzymes, tryptophan hydroxylase (TPH) and aromatic amino acid decarboxylase [1–4]. Serotonin is then released and exerts its various actions through serotonin receptors (5HTR) present on the cellular membrane [5–8]. In the nervous system the serotonin released into the synaptic cleft is transported back into the presynaptic neuron's cytosol by the serotonin transporter (SERT) [9,10]. Thus the three vital components of a functional serotonergic system are TPH, SERT and the 5HT receptor.

TPH catalyzes the rate-limiting step in the synthesis of serotonin; its expression is crucial for the activity of the serotonergic system [11,12]. They are of two types of TPH, TPH1 is mostly expressed in the periphery (skin, gut, pineal gland) but it can also be found in the central nervous system. TPH2 is exclusively expressed in neurons and is the predominant isoform of TPH found in the brain stem [1,13–15]. 5HT exerts both its CNS and non-CNS actions through seven distinct families of 5HT receptors (5HTR) 1–7. To date, 15 types of 5HT receptors have been described [5–8]. All 5HT receptors belong to the G-protein-coupled receptor (GPCR) superfamily, except for 5HTR3, which is a ligand-gated ion channel [5–8]. 5HT2A receptors are expressed abundantly in the CNS, especially at the serotonergic terminals. 5HT2A receptors are also expressed peripherally in platelets, vascular smooth muscle cells and the GI tract [16–20]. The 5HT transporter protein, SERT provides a mechanism for dynamic regulation of serotonergic activity. SERT has a common binding site for both 5HT and selective 5HT reuptake inhibitors (SSRIs), which bind specifically to the SERT and block the reuptake process. SSRIs, such as fluoxetine, are used in the treatment of mood disorders [9,10].

In the past two decades a lot of attention has been directed towards the role of serotonin in the regulation of mood and other CNS functions; however the majority of this monoamine is found outside the CNS [21]. Extra neuronal tissues have been shown to express all the fifteen types of serotonin receptors [21]. There are reports of local serotonergic systems in various tissues outside the CNS. These peripheral serotonergic systems perform vital regulatory functions in various organs outside the CNS. These data suggest that serotonin plays the role of a local hormone with a diverse set of functions, extra neuronally [21].

The 5HT induces its action on 5HT1B, 5HT2A, 5HT2B, 5HT4, and 5HT7 serotonin receptors on vascular smooth muscle cells. The predominant actions of 5HT on VSMCs include vasoconstriction, vasodilation, proliferation and migration involving different subset of 5HTRs [22–29]. The activation of 5HT2A, 5HT1B, and 5HT2B mediate vasoconstriction

or relaxation [26]. 5HT mediates its mitogenic action predominantly through the 5HT2A receptor. Activation of 5HT1B receptor induces vasoconstriction and mitogenesis, especially in the pulmonary vascular bed [30]. Recent research has documented the presence of functional SERT in aortic vascular smooth muscle cells of the rat [31,27] and demonstrated the critical role of SERT functionality in the mitogenic and co-mitogenic effects of 5HT [27].

Two major clinical studies, SADHART (Sertraline Anti-Depressant Heart Attack Trial) and ENRICHD (Enhancing Recovery In Coronary Heart Disease) showed an association between SSRI therapy and a decrease in mortality and cardiac morbidity in patients with CAD [32–34]. Other studies have also found an association between SSRI therapy and CAD mortality and morbidity. However, the underlying mechanisms are unknown and two major hypotheses are proposed [35]. In one of the hypotheses, 5HT released at the site of vascular injury, by platelets, is known to play an important role in the propagation of atherosclerosis and CAD by exacerbating mitogenic activity of VSMCs and vasoconstriction over mural thrombi [36,37]. Platelets store free serotonin in their cytosol by transporting it from blood, using SERT. SSRIs when administered inhibit SERT and decrease the concentration of serotonin in platelet cytosol, and this leads to a decrease in the amount of serotonin released at the site of vascular injury. This phenomenon has been claimed to be the important mechanism contributing to the decrease in incidence and severity of CAD in patients on SSRIs [35,38–42]. In the alternative hypothesis, pro-inflammatory cytokines that promote coronary atherosclerosis and CAD including IL-1, IL-6 and TNF-α are over expressed in depression [31,43]. IL-1, IL-6 and TNF-α stimulate vascular endothelial dysfunction and the evolution of coronary atherosclerosis, thus furthering the incidence of CAD [31,43]. SSRI therapy by alleviating depression is able to inhibit the overexpression of pro-inflammatory cytokines and thus decrease CAD incidence and severity.

Ni and colleagues [31] reported a functional serotonergic system in peripheral blood vessels with the presence of presence of TPH1 mRNA, and TPH protein in the vessel wall and functional TPH, SERT, and MAO-A in rat aorta and mesenteric arteries. Tissue samples of rat aorta (RA) and rat superior mesenteric artery (SMA) were demonstrated to possess functional TPH, functional SERT, and functional MAO-A. The authors demonstrated the presence of TPH1 mRNA, and TPH protein in the vessel wall [31]. These results reveal that there is evidence to support the argument of the presence of a peripheral serotonergic system within vascular smooth muscle cells. However the study by Ni et al does have its set of drawbacks [31]. The research was done on vascular tissue samples and not VSMC isolates, except for the immunocytochemical studies [31]. It could be argued that the functional activity of serotonin synthesis, uptake and metabolism, which was demonstrated in the study, was due to the platelets, mast cells, vascular endothelial cells and sympathetic nerve endings within the tissue and not from the VSMCs [31].

Here, we propose the presence of a peripheral serotonergic system within human coronary arterial VSMCs (HCASMC) that produce their own serotonin to act in an autocrine and paracrine fashion to augment VSMC mitogenesis. If it is true, the SSRI therapy would then be able to inhibit this intrinsic serotonergic system of VSMCs, as it would decrease the entry of serotonin into the HCASMCs and inhibit HCASMC mitogenesis. This process would

decrease the evolution of the coronary atherosclerosis and hence decrease the mortality and morbidity in CAD. This would also mean that serotonin induced HCASMC mitogenesis is not exclusively dependent on its release from platelets. The findings from this study could have far reaching clinical and translational ramifications, and would be a strong argument for the initiation of SSRI therapy in patients with CAD or CAD risk factors and co-existent depression, and it could pave the way for studies on innovative clinical interventions such as SSRI eluted coronary stents.

Materials and Methods

Reagents

The cell culture media, growth supplements, antibiotics, amphotericin were purchased from Science Cell (Carlsbad, CA), and fetal bovine serum was purchased from Gibco (Carlsbad, CA). The following antibodies were used: TPH1 (ab52954) (Abcam, Cambridge, MA), SERT (H00006532-D01P) and GAPDH (NB300-221) (Novus Biologicals, Littleton, CO). The following positive controls were used for the antibodies: SERT overexpression 293T cell lysate (H00006532-T01) (Novus Biologicals, Littleton, CO) and THP1 whole cell lysate (ab7913) (Abcam, Cambridge, MA).

Cell culture

HCASMCs were obtained from Cell Applications (San Diego, CA) at passage 3. Cells were grown in smooth muscle cell media (SMCM) supplemented with 10% heat inactivated FBS, smooth muscle cell growth supplement and 1% penicillin-streptomycin-amphotericin. Confluent HCASMCs (about 75–80%) were harvested by trypsinization and were used for isolation of protein, mRNA, and genomic DNA.

HEK-293 cells stably transfected with hSERT cDNA (HEK-hSERT) [44] were obtained from Dr. Randy Blakely (Vanderbilt University), for use as positive controls. HEK-hSERT cells from passage 15–20 were used in the experiments. The cells were cultured in high glucose DMEM (4.5 g/L of dextrose; Sigma, St. Louis, MO), with dialyzed 10% FBS (Sigma), penicillin & streptomycin (Sciencell Research Laboratories), 1% glutamine (Gibco) and 250 μg/ml geneticin (Life Technologies, Grand Island, NY). Confluent cells (75 – 80%) were harvested by trypsinization and were used for experiments.

The NCI-H727 cells (human bronchial carcinoid cell line) (ATCC, Manassas, VA) were used as cellular positive controls for TPH1 experiments. The culture media for NCI-H727 cells was ATCC-formulated RPMI-1640 Medium, with 10% heat-inactivated FBS with penicillin and streptomycin (Sciencell Research Laboratories). Confluent cells (75 – 80%) were harvested by trypsinization and used for experiments.

RNA isolation and quantitative PCR

The cells were grown to 75 to 80% confluence and total RNA was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany). The yield of RNA was quantified using Nanodrop (Thermo Scientific, Rockford, IL). First-strand cDNA synthesis was done using 1 μg total RNA with oligo dT (1 μg), 5X reaction buffer, $MgCl_2$, dNTP mix, RNAse inhibitor, and

Improm II reverse transcriptase as per Improm II reverse transcription kit (Promega, Madison, WI). After the first strand synthesis, real-time PCR was done using 1 μg cDNA, 10 μl SYBR Green PCR master mix (Applied biosystems, Life technologies, Carlsbad, CA), forward, and reverse primers (10 pmol/μl) (Integrated DNA Technologies, Coralville, IA) using a CFX 96 - real-time PCR system (Bio-Rad, Hercules, CA). The primer sequences are given in Table 1. The specificity of the primers was analyzed by running a melt curve. The PCR cycling conditions used were 5 min at 95°C for initial denaturation, 40 cycles of 30 s at 95°C, 30 s at 58°C, and 30 s at 72°C. Each real-time PCR run was carried out in triplicates and the threshold cycle values were averaged. Calculations of relative gene expression were based on the differences in the threshold cycles. The fold change in expression between samples was calculated by fold change = $2^{-\Delta\Delta Ct}$ method [45]. The results were normalized to 18S rRNA expression.

Agarose gel electrophoresis and DNA sequencing

Real-time PCR products were subjected to agarose gel electrophoresis and subsequently sequenced to verify the sequence homology of the amplified genes, using NCBI BLAST resource. Briefly, a 2% Agarose gel was casted by adding 900 mg agarose to 45 ml of 1xTAE buffer and microwaved until the agarose was fully dissolved. The mixture was then allowed to cool down, to about 50°C and 5 μl of Lonza gel star staining solution (10,000X) was added to the mixture. The dissolved agarose solution was poured into the gel boat with gel combs positioned and the gel was allowed to polymerize at room temperature for 45 mins. After polymerization, 10 μl of 100bp DNA ladder marker (NEB N3231S) was added into the reference well at one end. The qPCR products from each PCR reaction were then loaded into each well of the above 2% agarose gel and electrophoresis was carried out at 5 volts/cm for about 2 hours. The gel was viewed under an UV illuminator in the ChemiDoc MP imaging system (Bio-Rad) and the image at 300 dpi was captured using live camera.

The amplified bands of interest were carefully excised from the agarose gel, under UV light illumination and DNA was extracted from the gel using QIAquick Gel Extraction Kit (Qiagen), as per the provided protocol in the product manual. The eluted DNA was sequenced using the same primers that were used in the above qPCR. Briefly, about 12 ng of the eluted amplicon from the gel was mixed with 12.8 pmol of primer and the total volume of the mixture was adjusted to 20 μl with sterile water. Separate sequencing reaction mixtures were made with both forward and the reverse primers. This DNA primer mix was subjected to sequencing and the chromatograms obtained from the DNA sequencer are shown.

Western blot analysis

The cells were suspended in a radioimmunoprecipitation assay buffer (Sigma, St Louis, MO) containing 25 mM Tris·HCl (pH 7.6), 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, and 0.1% SDS for 30 min on ice with protease inhibitor (Sigma) and were intermittently mixed. Suspended cells were centrifuged at 12,000 rpm for 15 min. Supernatant was used for protein analysis. The protein content of the sample was measured using bicinchoninic acid assay (BCA) protein assay kit according to the manufacturer's protocol (Sigma). The protein samples (30 μg) were subjected to 10% SDS-PAGE (Bio-Rad,

Hercules, CA) and then transferred to nitrocellulose membrane (Bio-Rad) for immunoblotting. After the nonspecific proteins were blocked with 5% milk, the membrane was washed and incubated overnight at 4°C with targeted antibodies, which were diluted in 1:500 in nonfat milk with PBS-Tween. The blot was washed again and incubated for 1 hour with secondary antibody, horseradish peroxidase-conjugated anti rabbit (Novus Biologicals) diluted at 1:1,000. Finally, the immunoblot was developed with ECL chemiluminescence detection reagents (Amersham Pharmacia Biotech) system using UVP Bioimaging system. Results were normalized against the expression of the reference protein GAPDH (glyceraldehyde-3-phosphate dehydrogenase). Densitometric analysis for quantification of the relative protein expression was performed using ImageJ software developed for Mac OSX by NIH.

Serotonin transporter uptake assay

In cells serotonin transporter activity was detected with Neurotransmitter Transporter Uptake Assay Kit (Molecular Devices, LLC, Sunnyvale, CA, R8173). It is a fluorescence-based assay employing a fluorescent substrate that mimics serotonin, which is taken into the cell through serotonin transporter, resulting in increased intracellular fluorescence. The fluorophores not transported into the cell (extracellular) bind to the masking dye in the kit reagent. The fluorescence intensity of the free, serotonin-mimicking, fluorophores within the cell is a function of the activity of the serotonin transporters expressed on the cell surface.

The cells were seeded in a 96 well microtiter plate at a density of 40,000 cells/well in a volume of 100μL as recommended in the protocol provided with the kit. Four of the wells were not seeded with any cells and they served as blanks in the experiment. Half of the wells (except for the four blank wells) were seeded with HCASMCs while the other half were seeded with HEK-hSERT cells. Then, the cells were allowed to adhere and acclimate to the plate for 24 hours prior to assaying as per the protocol. The wells containing cells were split into two equal groups, control and the test. The grouping was made in such a way that both the groups had equal number of HCASMCs, and HEK-hSERT cells. In order to examine the specificity of SERT activity, an inhibitor of of SERT, fluoxetine (100μg/ml), was used. Then, the plates were incubated at 37°C for 30 minutes, to allow the binding of fluoxetine to the transporter. The 100μL of 1X HBSS was added to two of the blank wells, whilst 100μl of dye solution was added to the other two. The plate was then directly transferred to the EnSpire® Multimode Plate Reader (Perkin Elmer, Waltham, MA) and incubated for a further 10 min at 37°C and then read at endpoint read mode.

TPH enzymatic activity by UPLC

To determine the enzymatic activity of TPH, we adapted the technique and protocol employed by Hasegawa et al., 1999 [46]. NCI-H727 bronchial carcinoid cells served as positive controls.

Trypsinized HCASMCs and control cells were first disrupted by two cycles of freezing and thawing at 5 min intervals and to the cell lysate thus obtained, from each T-25 corning flask, 20 μL of (Ca2+/Mg2+minus) PBS was added. To this cell lysate 50ul of the pre incubation cocktail was added. This pre incubation buffer consisted of 50 μM ferrous ammonium

sulfate (Sigma), 30mM dithiothreitol (DTT) (Sigma), catalase (4 mg/mL, bovine liver; Sigma), and Tris/acetate (Sigma) (pH 8.1) at a concentration of 0.1M in a total volume of 50 mL. Then 100 μL of the incubation cocktail was added and the mixture was incubated at 30°C for an additional 10min to initiate the reaction. The incubation mixture was prepared by adding 0.25mM L – tryptophan, 0.4 mM (6R)-l-erythro-tetrahydrobiopterin, 0.5 mM NADH (nicotinamide adenine dinucleotide, 1 mM NSD-1015 (aromatic amino acid decarboxylase inhibitor/3-Hydroxybenzylhydrazine dihydrochloride; Sigma), 4.5 μg/mL dihydropteridine reductase from sheep liver (Sigma), 2 mg/mL catalase (Sigma), and 0.1M potassium phosphate (pH 6.9) (Sigma) [47]. NSD 1015 is added to aid in the accumulation of 5-hydroxy tryptophan the direct product of TPH's enzymatic activity.

The reaction was quenched with 10 μL of 40% trichloroacetic acid. The samples were then centrifuged at 12,000g for 10 min to pellet precipitated protein. The supernatant was then subjected to UPLC to detect the presence of 5-hydroxy tryptophan. The mobile phase was a 90:7:5 mixture of 40 mM of sodium acetate (pH adjusted to 3.5 with formic acid: Sigma), acetonitrile and methanol at a flow rate of 1 mL/min. Fifty microliters of the supernatant from the reaction mixture was injected into the Waters ACQUITY UPLC system equipped with a quaternary solvent delivery manager and a sample manager (Waters Corporation, Milford, MA). Chromatographic separations were performed on a 2.1 × 50 mm 1.7 μm ACQUITY BEH C18 chromatography column (Waters Corporation, Milford, MA). Tryptophan (Sigma), and 5-hydroxytraptophan (Sigma) were used as chemical standards for the UPLC experiment.

Results

Melting curves for TPH1 and SERT mRNA transcripts

The melt curve of the qPCR products (temperature in Celsius vs. the first negative derivative of the rate of change of fluorescence (-d (RFU)/dT)) derived from HCASMCs and positive controls (HEK-hSERT), using the primer pair S2 (primers used to amplify sequences within the SERT gene), displayed sharp peaks (Figure 1a). The sharp melt peaks of the qPCR products derived from the HCASMCs and HEK-hSERT cDNA confirmed the specificity of the S2 primer pair, and that the qPCR run amplified only amplicons of a particular nucleotide length and sequence. The melt curve of the qPCR products derived from HCASMCs and positive controls (NCI-H727), using the primer pair T3 (primers used to amplify sequences within the TPH1 gene), displayed sharp peaks (Figure 1b). The sharp melt peaks of the qPCR products derived from the HCASMCs and NCI-H727 cDNA, confirmed the specificity of the T3 primer pair, and that the qPCR amplified only amplicons of a particular nucleotide length and sequence particular amplicon.

TPH1 and SERT mRNA transcripts in HCASMCs

The cumulative data from several independent samples comparing the mRNA transcripts of SERT and TPH1 between HCASMCs and their respective positive controls is shown in Figure 1c and 1d. There was no significant difference in the normalized expression of SERT between HCASMCs and HEK-hSERT (Figure 1c). However, a relatively higher expression of TPH1 in NCI-H7272 compared to HCASMCs (Figure 1d).

Confirmation of primer pair specificity

The qPCR products amplified from the total RNA derived from HCASMCs, using S2 pair of primer, was subjected to 2% agarose gel electrophoresis. It formed a single band at just above the 200bp mark as expected, thus confirming the specificity and selectivity of the primer pair. The exact expected amplicon size from the primer pair S2 is 221bp. Though S1 and S2 primer pairs were used in the first qPCR run, to check primer specificity, we used S2 primer pair for our qPCR experiments. The image obtained from the gel is shown in Figure 2a. The qPCR products amplified from the total RNA derived from HCASMC cells, using T3 pair of primer, produced a single band between the 100bp and 200bp as anticipated. The exact expected amplicon size from the T3 primer pair was 143bp. The image obtained from the gel is shown in Figure 2b.

The specificity of the primer pairs was further confirmed by gene sequencing. The bands from the DNA gel electrophoresis of the qPCR products were eluted and sequenced. The DNA sequencing was done to confirm the amplicons obtained from HCASMC cDNA, using the S2 and T3 primer pairs. The sequences obtained aligned with SERT and TPH1 genes (the target genes of interest). The sequence of the amplicons and the gene sequencing chromatogram are shown in Figures 2c–2f. The alignment of the DNA sequences of the amplicons with SERT (SLC6A4 gene) and TPH1 gene sequence was evaluated using Basic Local Alignment Search Tool. The sequence of the amplicons derived from HCASMC cDNA templates, using S2 primer pair and the T3 primer pair, was aligned with 100% homology with SLC6A4 mRNA and TPH1 mRNA, respectively.

HCASMCs express TPH1 and SERT protein

There were detectable levels of TPH1 and SERT protein in HCASMCs. Blots showing the band at the expected molecular weights are displayed in Figure 3a and Figure 3b. The cumulative densitometric analyses from separate experiments revealed significant protein expression of both TPH1 and SERT in HCASMCs (Figure 3c).

SERT activity in HCASMCs

The SERT activity was measured by SERT endpoint assay utilizing fluorescence intensity emitted at the end of the reaction (Figure 4). In all samples of HCASMCs and HEK-hSERT cells, strong SERT activity was found. The SERT activity was inhibited by 100μg/ml fluoxetine, supporting the specific activation of SERT in HEK-hSERT cells (Figure 4).

TPH activity in HCASMCs

By UPLC we confirmed the presence of 5-hydroxy tryptophan, the product of TPH enzymatic reaction. The retention times of tryptophan and 5-hydroxytraptophan standards were 1.008 min and 0.567 min, respectively, in HCASMCs. The chromatograms that depict the retention times of the standards are shown in Figure 5a and Figure 5b. In all the 6 HCASMC samples, the reaction product 5-hydroxy tryptophan and the substrate tryptophan were both detected as peaks in the UPLC chromatogram at a retention time of 0.5203(± SEM 0.0021) min and 1.2103 (± SEM 0.0037) min respectively. In NCI-H727 (positive controls) samples 5-hydroxy tryptophan and tryptophan was detected as peaks in the UPLC chromatogram at 0.5223(± SEM 0.0027) min and 1.2092 (± SEM 0.0031) min, respectively.

The UPLC chromatograms that show the presence of 5-HTP (5-hydroxy tryptophan) in the HCASMC and NCI-H727 samples are shown in Figure 5c and Figure 5d. The detection of 5-hydroxy tryptophan in the reaction supernatant demonstrates TPH activity in the HCASMCs and NCI-H727 cells.

Discussion

Coronary artery diseases are one of the major causes of morbidity and mortality, both in the US and worldwide. Many patients who are either afflicted with or prone to CAD are affected by major depression, as co-morbidity [48]. This subset of patient population is treated with various anti-depressants, including SSRIs, to ameliorate their symptoms of depression [48]. Studies have shown a decrease in the severity and incidence of CAD associated with SSRI therapy in patients with CAD [48,35,38,39]. Many mechanisms have been postulated to be responsible for this association. It includes the decrease in serotonin released by platelets at the site of a coronary atherosclerotic plaque, down regulation of inflammatory cytokine secretion by SSRIs in depression [35], and the effects of SSRI on autonomic regulation of cardiac functions [35]. However, none of these hypotheses have been substantiated with concrete evidence.

In 2008, Ni and colleagues outlined the probable existence of a serotonergic system within the peripheral arterial wall, particularly, within the VSMCs of the arterial media of the rat aorta and superior mesenteric artery [31]. This encouraged us to hypothesize that there is a serotonergic system in HCASMCs. By down regulating this system, SSRIs could retard the progression of coronary atherosclerosis and hence cause a decrease in the severity and incidence of CAD. To substantiate this hypothesis, it is critical to first demonstrate the presence and functionality of the essential components of the serotonergic system within HCASMCs. Hence, we designed a research study to examine the presence of functional SERT and TPH, two critical components of the serotonergic system, in HCASMCs.

We found significant expression of both the protein and mRNA transcripts of TPH, and SERT suggesting the presence of a serotonergic system in HCASMCs. Moreover based on our serotonin reuptake assay and TPH enzymatic reaction by UPLC, we were also able to demonstrate that the TPH and SERT found in the HCASMCs are functional.

The presence of various 5HT receptors on HCASMCs, the other branch of the effector arm of the serotonergic system, has been documented in many investigations [26,49]. Taken together with our findings one could say that HCASMCs possess both the synthetic and effector arms of a serotonergic system. This intrinsic serotonergic system could be regulated just like any other serotonergic system elsewhere in the body, as it possesses the same basic components, SERT, and TPH. Pharmacological substances, such as SSRIs, would be able to interact with the SERT regulating internalization of serotonin and hence its ability to participate in serotonylation within HCASMCs. These local systems, though miniscule, might possess considerable physiological significance. In the case of serotonin, an increase in its blood concentration does not always correlate with essential hypertension or atherosclerosis, given its low half-life in the extracellular milieu. However intracellular serotonin if present could be concentrated by SERT and be used in a paracrine fashion by

cells that possess the required machinery to do so. To demonstrate in detail the way in which the internalized serotonin in HCASMCs produce cell proliferation more studies have to be done.

This study should pave the way for further studies to evaluate the effect of SSRIs in cardiovascular system, especially its effect on HCASMC mitogenesis. Further in vitro studies that document the effects of the intrinsically produced serotonin's proliferative action on HCASMCs are required. This study has immense translational value, as it would help in defining clinical recommendations, which shall include SSRI therapy, in patients with coexistent affective disorders and CAD or risk factors for CAD. It might also initiate further research in other therapeutic modalities that could explore this pathophysiological association in treating and preventing coronary atherosclerosis.

There are few potential limitations and warrants further studies. The direct secretion of serotonin from HCASMCs has not been demonstrated. Although we demonstrated the presence of mRNA transcripts and protein of TPH, the enzymatic activity of aromatic amino acid decarboxylase is yet to be examined. We did not study the direct effect of the serotonergic system on the mitogenesis of HCASMCs, which is a hallmark of intimal hyperplasia and coronary atherosclerosis.

Conclusion

Thus, our findings support the presence of SERT and TPH1 in HCASMCs together with the functionality of the transporter and the presence of TPH1, the rate-limiting enzyme in the synthesis of serotonin. Since the presence of 5HT receptors, MAO-A and amino acid decarboxylase in HCASMCs have been shown by other investigators [50,31,51–56], we, for the first time, report the existence of a functional local serotonergic system equipped with the machinery to synthesize and concentrate serotonin in HCASMCs. We propose that this system plays an important role in VSMC proliferation, migration and atherosclerosis. Obviously, additional studies are required to investigate the mitogenic effects of the intrinsically secreted serotonin on HCASMCs.

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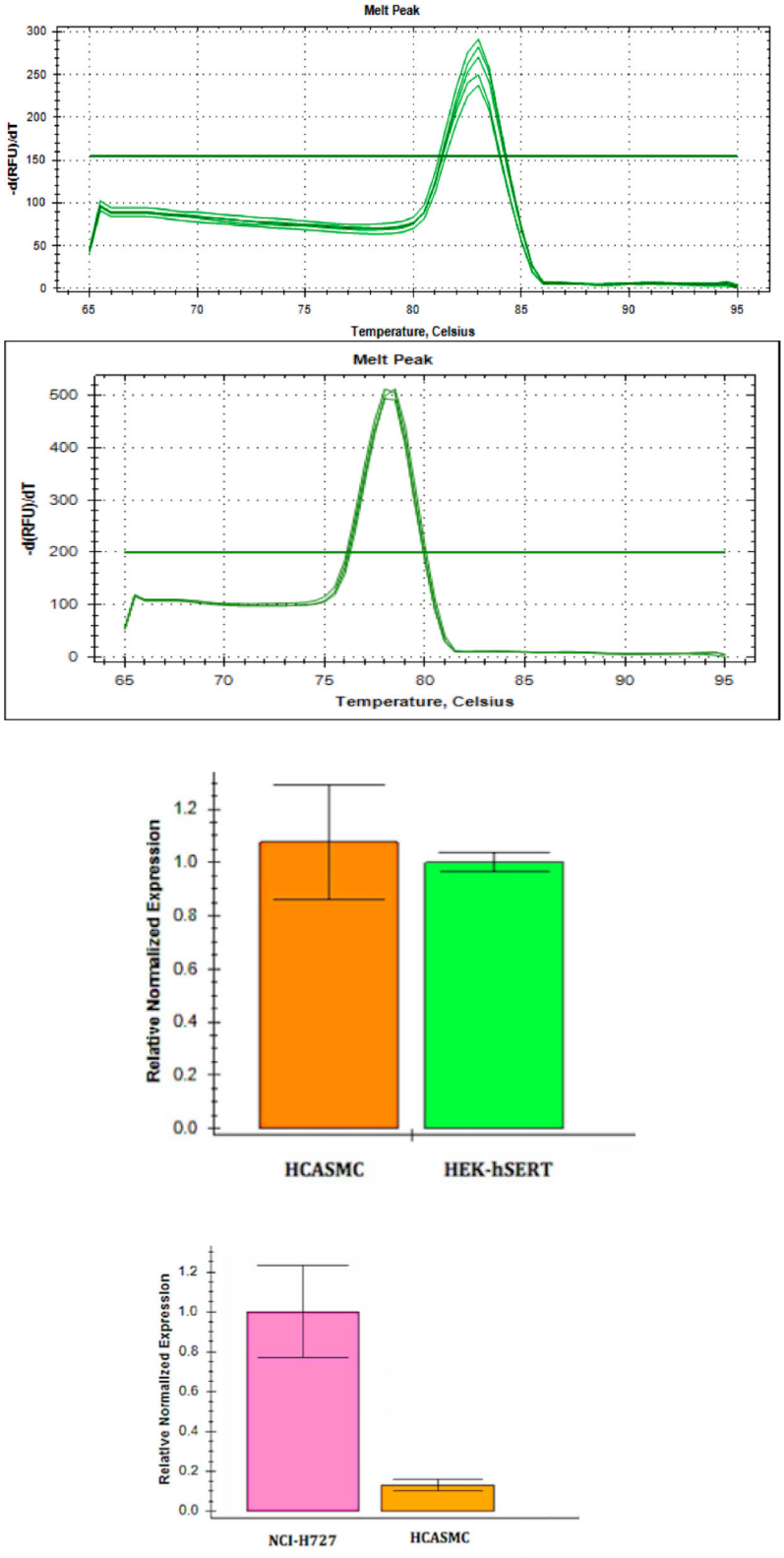
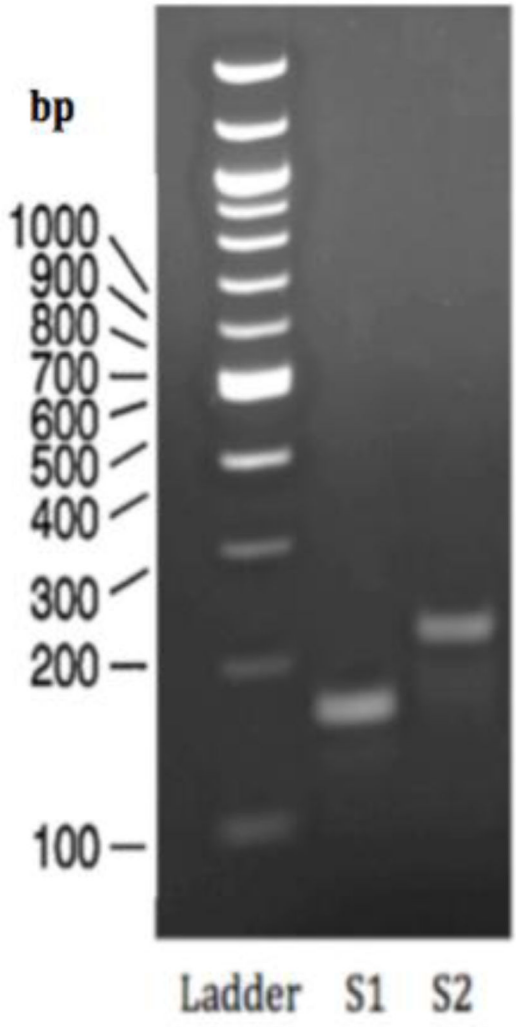
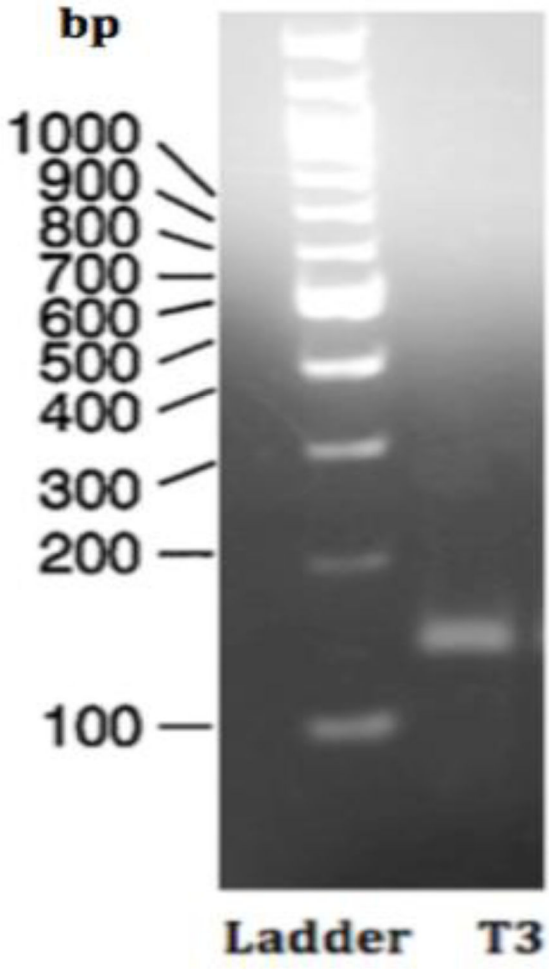
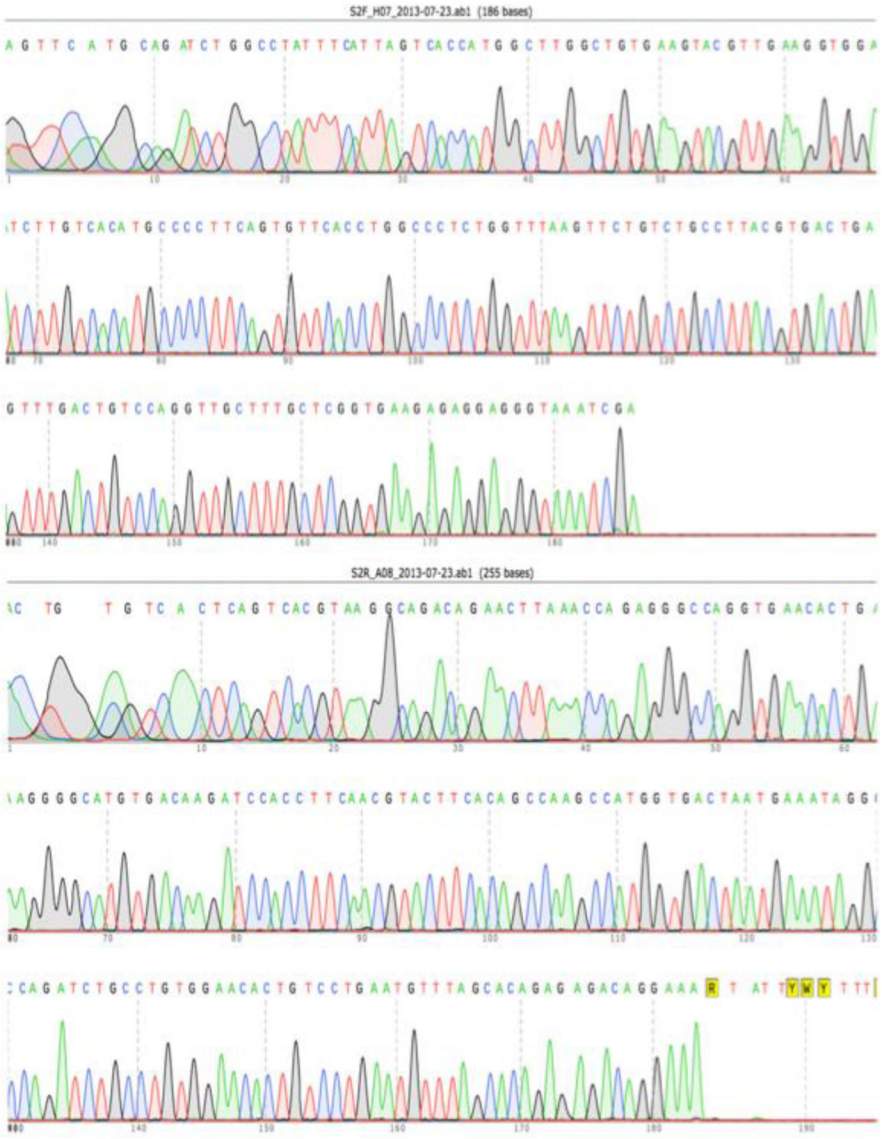


Figure 1. Representative melt curves and relative mRNA transcripts of SERT and TPH1

Representative multiple real-time PCR runs are shown in Figure 1a and Figure 1b. The melt curve is plotted with temperature in Celsius vs. the first negative derivative of the rate change of fluorescence (-d(RFU)/dT). (a) The sharp melt peaks of qPCR products derived from the cDNA of HCASMCs and HEK-hSERT cells, amplified using S2 primers (SERT primers) is shown, indicating the specificity of the S2 primer pair. (b) The sharp melt peaks of qPCR products derived from the cDNA of HCASMCs and NCI-H727 cells, amplified using T3 primer pair (TPH1 primers) is shown, indicating the specificity of the T3 primer pair. (c) The bar diagram shows the mean ± SEM of relative normalized SERT mRNA expression in HCASMCs and HEK- hSERT cells. (d) The bar diagram shows the mean ± SEM of relative normalized TPH1 mRNA expression in HCASMCs and NCI- H727 cells. The relative expression of SERT or TPH1 was normalized against the expression of the reference gene18S rRNA, using $2^{-\Delta\Delta}$Ct method (N=6 in each experimental group). (N=6).







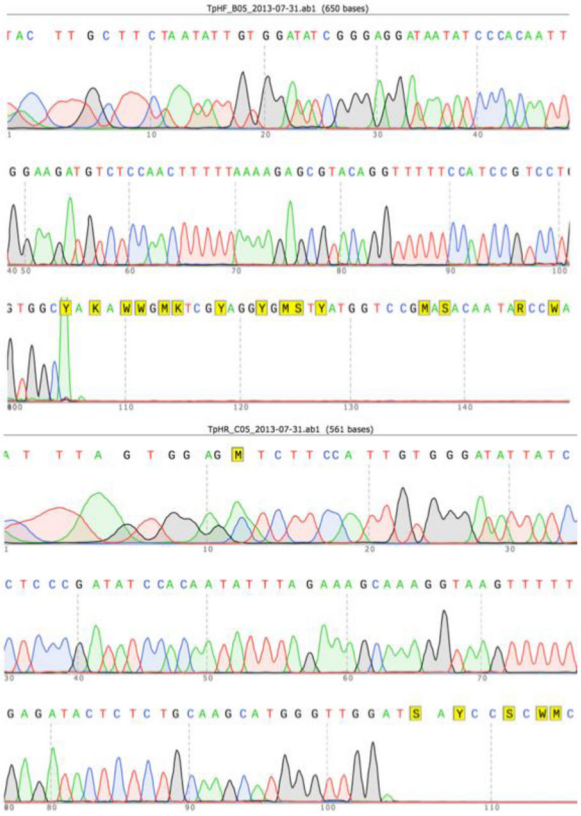


Figure 2. DNA gel electrophoresis of qPCR products and sequencing of the eluted product as shown by chromatogram out put

(a) SERT primers: a single band between 200 and 300bp (expected 221bp) was obtained in the qPCR amplification using both S1 and S2 primer pairs. Only S2 primer pair was used for qPCR experiments. (b) TPH1 primer: a single band was obtained, with a length between 100 and 200bp (expected 143bp). (c) The figure illustrates the sequence corresponding to SERT gene, as obtained when the forward primer of S2 primer pair was used with the amplified DNA. (d) The figure illustrates the sequence corresponding to SERT gene, as obtained when the reverse primer of S2 primer pair was used with the amplified DNA. (e) Figure illustrates the sequence corresponding to TPH1 gene, as obtained when the forward primer of T3 primer set was used with the amplified DNA. (f) Figure illustrates the sequence corresponding to TPH1 gene, as obtained when the reverse primer of T3 primer pair was used with the amplified DNA.

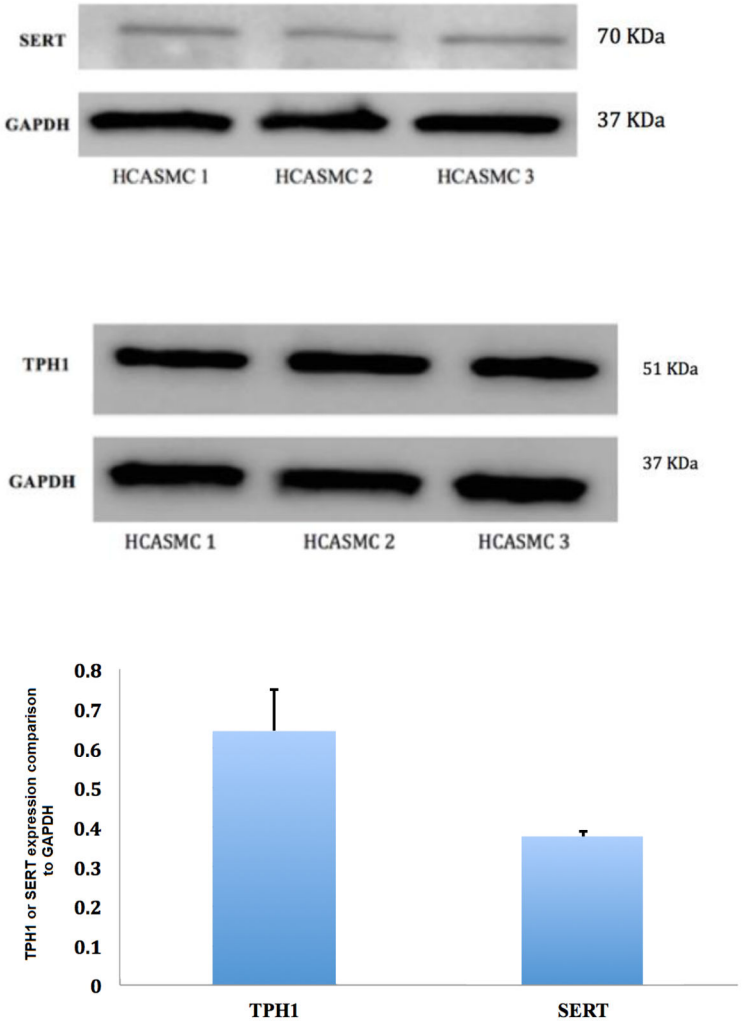


Figure 3. Expression of SERT and TPH1 protein in HCASMC

Expression of SERT (a) and TPH1 (b) protein derived from HCASMC at 70 kDA and at 51kDa, respectively. The bar chart (c) depicts the optical density of SERT and TPH1 normalized to GAPDH. Each bar represents mean ± SEM from 6 independent experiments. (N=6).

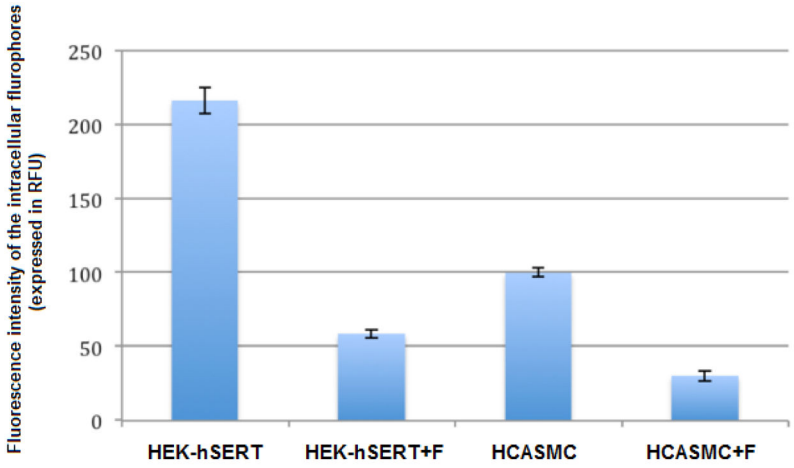
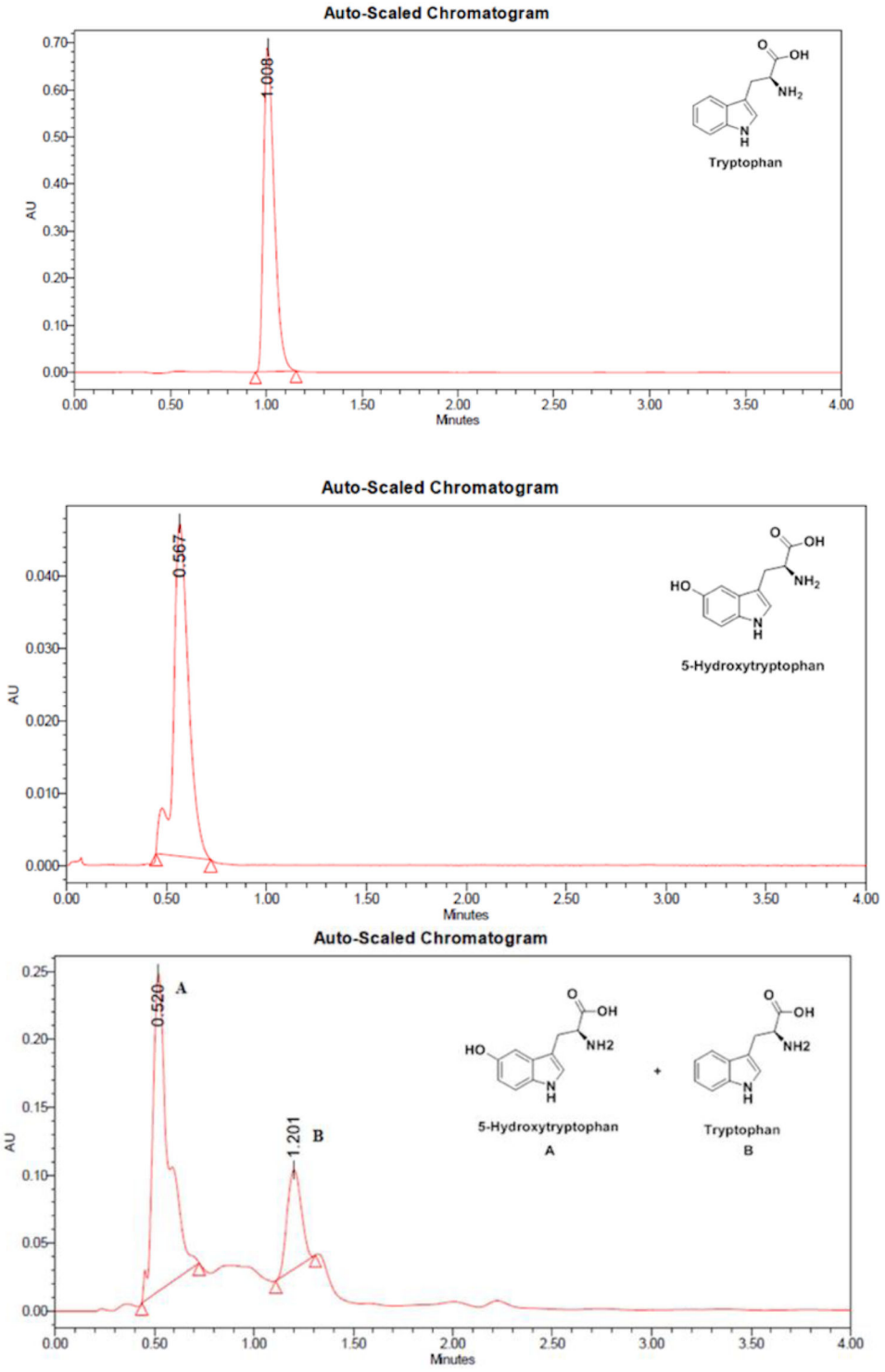


Figure 4. Endpoint assay to measure SERT activity

The results of the endpoint assay obtained as florescence reading on the plate reader is displayed as a bar graph depicting the fluorescence intensity emitted at the end of the reaction from 6 different samples of HCASMCs and HEK-hSERT cell groups. The endpoint assay illustrates the presence of SERT activity in both HCASMCs and HEK-hSERT cells. This activity is inhibited by fluoxetine, causing a relative decrease in fluorescence. Data shown is mean ± SEM of the RFU. (HSERT, HEK-hSERT cells; F – Fluoxetine) (N=6).



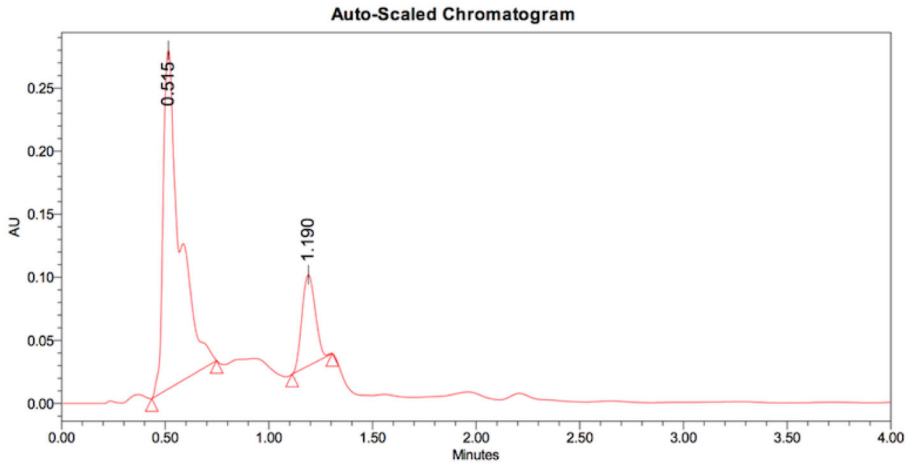


Figure 5. UPLC Chromatogram of TPH reaction in HCASMC

The chromatogram (a) shows a sharp peak at 1.008 minutes, which is the retention time of tryptophan standard. The chromatogram (b) shows a sharp peak at 0.567 minutes, which is the retention time of 5-HTP standard. The chromatogram (c) illustrates peak A at retention time of 0.520 min which is 5-HTP and peak B at a retention time of 1.201 which is tryptophan in the HCASMC reaction sample. The chromatogram (d) illustrates two peaks the first one from the left corresponds to a retention time of 0.520 min, which is 5-hydroxy tryptophan and second peak from the left with a retention time of 1.201 is tryptophan in the NCI-H727 reaction sample. (N=6).

Table 1

The sequence of the primers used in real time PCR

Gene of interest	Primers (Integrated DNA Technologies)
SLC6A4 (serotonin transporter gene)	Forward primer (5′ – 3′): TTCCTGTCTCTCTGTGCTAAAC
	Reverse Primer (5′ – 3′): CGATTTACCCTCCTCTCTTCA
TPH1 (Tryptophan hydroxylase 1 gene)	Forward primer (5′ – 3′): CCAACCCATGCTTGCAGAGA
	Reverse Primer (5′ – 3′): GCCACAGGACGGATGGAAAA
18S (18S ribosomal RNA)	Forward primer (5′ – 3′): TCAACTTTCGATGGTAGTCGCCGT
	Reverse Primer (5′ – 3′): TTCTTGGATGTGGTAGCCGTTTCT