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Characterization of Serotonin and *N*-acetylserotonin Systems in the Human Epidermis and Skin Cells

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Abstract

Tryptophan hydroxylase (TPH) activity was detected in cultured epidermal melanocytes and dermal fibroblasts with respective Km of 5.08 and 2.83 mM and Vmax of 80.5 and 108.0 μmol/min. Low but detectable TPH activity was also seen in cultured epidermal keratinocytes. Serotonin and/or its metabolite and precursor to melatonin, *N*-acetylserotonin (NAS), were identified by LC/MS in human epidermis and serum. Endogenous epidermal levels were 113.18 ± 13.34 and 43.41 ± 12.45 ng/mg protein for serotonin (n=8/8) and NAS (n=10/13), respectively. Their production was independent of race, gender and age. NAS was also detected in human serum (n=13/13) at a concentration 2.44 ± 0.45 ng/mL, while corresponding serotonin levels were 295.33 ± 17.17 ng/mL (n=13/13). While there were no differences in serum serotonin levels, serum NAS levels were slightly higher in females. Immunocytochemistry studies showed localization of serotonin to epidermal and follicular keratinocytes, eccrine glands, mast cells and dermal fibrocytes. Endogenous production of serotonin in cultured melanocytes, keratinocytes and dermal fibroblasts was modulated by UVB. In conclusion, serotonin and NAS are produced endogenously in the epidermal, dermal and adnexal compartments of human skin and in cultured skin cells. NAS is also detectable in human serum. Both serotonin and NAS inhibited melanogenesis in human melanotic melanoma at concentrations of 10^{-4}-10^{-3} M. They also inhibited growth of melanocytes. Melanoma cells were resistant to NAS inhibition, while serotonin inhibited cell

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growth only at 10^{-3} M. In summary, we characterized a serotonin-NAS system in human skin that is a part of local neuroendocrine system regulating skin homeostasis.

Keywords

epidermis; serotonin; *N*-acetylserotonin; skin cells

Introduction

Human skin composed of the epidermis, dermis and hypodermis (subcutaneous fat adjacent to dermis also called as subcutis) is the largest body organ that not only serves as the protective barrier [1,2] but is also empowered with sensory and regulatory functions necessary for organismal survival [3]. Thus, it has the capability to sense and adapt to environmental changes and to regulate and protect local and central body homeostasis [3]. These functions are coordinated by the cutaneous neuroendocrine system [4–6]. The epidermis represents the most-outer layer of the skin that builds a protective barrier against environmental stressors including ultraviolet radiation (UVR)[7] through differentiation of keratinocytes and their interaction with melanocytes [1,2,8].

Serotonin (5HTP), a product of sequential metabolic transformation of L-tryptophan (Trp) →hydroxyTrp→5HTP is produced across different species representing an ancient molecule with pluripotent and diverse activities [9–13]. The rate-limiting step in serotonin synthesis is tryptophan hydroxylation at position 5 on the indole ring to produce 5-hydroxytryptophan (TrpOH) in a reaction catalyzed by tryptophan hydroxylase and requiring molecular oxygen and the reducing cofactor 6-tetrahydrobiopterin [14–16]. There are two isoforms of the enzyme encoded by separate genes. *TPH1* expression is the highest in the pineal gland [15], while being detected in different body organs and cells types including skin [16], and *TPH2* expressed predominantly in neuronal cells and representing the main isoform in the brain [17]. Both isoenzymes are expressed in the human skin with *TPH2* being predominantly expressed in normal and malignant melanocytes and retinal pigment epithelium [18–21]. TrpOH can also be produced in the skin through H_2O_2- and ultraviolet A (UVA) irradiation induced a free-radical-mediated oxidation of L-tryptophan [22].

Serotonin is also a precursor to melatonin, a molecule with diverse biological activities [9,23,24], formed through sequential acetylation to *N*-acetylserotonin (NAS) by either arylalkylamine *N*-acetyltransferase/serotonin *N*-acetyltransferase (AANAT/SNAT) [23–25] or arylamine *N*-acetyltransferase (NAT) with mixed arylamine/arylalkylamine substrate specificity [26–28], and following methylation by hydroxyindole-*O*-methyltransferase/*N*-acetylserotonin methyltransferase (HIOMT/ASMT) [23,24,29]. All these enzymes are expressed in the human skin [18,30]. NAS is also a product of melatonin metabolism by CY1B1 [23], an enzyme also expressed in the human skin [31,32]. Thus, in the skin serotoninergic and melatoninergic pathways are tightly interconnected with NAS representing a precursor to and a product of melatonin metabolism [18,33].

Serotonin regulates a wide range of physiological and pathological processes in humans predominantly through interaction with cell surface membrane-bound G protein coupled

receptors or ligand-gated ion channels classified into seven general families (5HTR1–7R) [10–12,34,35]. They can attenuate (5HTR1/5) or stimulate (5HTR4/6/7) adenylate cyclase activity or enhance production of inositol-triphosphate (IP3) and diaglycerol (DAG) (5HTR2AR) or function as an ion channel (5HTR3R) [34,35].

Human skin or skin cells express genes and proteins of 5HTR1A, 1B, 2A, 2B, 2C, 3 and 7 receptors, whose activation would contribute to the pleiotropic effects of serotonin on skin functions including vascular and inflammatory responses, wound healing and potentially contributing to regulation of hair cycling and melanin pigmentation [36–41]. However, there is a paucity of information on the biological role of NAS except its contribution to photoprotection against UVB [42]. In addition, it is unclear whether NAS is only produced locally or whether it can enter the systemic circulation. Therefore, we decided to characterize 5HT/NAS systems in the human skin.

Materials and Methods

Chemicals

Charcoal stripped fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Lawrenceville, GA, USA). Serotonin and *N*-acetylserotonin were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade acetonitrile, water and acetic acid (Fisher scientific, Pittsburgh, PA, USA) were used for HPLC. For LC-MS system, acetonitrile, water and formic acid (Sigma-Aldrich, St. Louis, MO, USA) were used. Dulbecco's modified Eagle's medium (DMEM) with high glucose (4,500 mg/L), 1% penicillin-streptomycin solution (10,000 units of penicillin and 10 mg of streptomycin in 1 mL 0.9% NaCl), DMSO, ethanol, HEPES (1 M), L-3,4-dihydroxyphenylalanine (L-DOPA), NaOH, non-essential amino acids (NEAA) (100×), RPMI-1640 medium, serotonin, sodium pyruvate (100 mM), Triton® X-100, were purchased from Sigma-Aldrich (St. Louis, MO, USA). FBS, 0.05% trypsin/0.53 mM EDTA solution, 1×PBS (pH 7.4), L-glutamine (200 mM) were supplied by Thermo Fisher Scientific (Waltham, MA, USA). Deuterated standard of *N*-acetylserotonin [*N*-acetylserotonin-d_7 (NAS-D_7)] was received from the National Institute of Mental Health Chemical Synthesis and Drug Supply Program NIMH # A-906, Bethesda, MD, USA.

Human skin and serum

The use of human tissues was approved by both UTHSC and UAB Institutional Review Boards as an exempt protocol #4 and collected as described previously [43,44]. The skin specimens (n=13) were obtained from both males and females [30 to 90 years old] of African-American and Caucasian races and the epidermis was peeled and extracted with 75% acetonitrile with homogenization as described previously [45–47]. Serum samples were collected from thirteen healthy adult volunteers (12 Caucasians and 1 Hispanic, with an age range of 25 to 61 years including 10 females and 3 males) according to the IRB protocol #7526 (Dr. A. Postlethwaite, P.I.) as described previously [43,44]. The samples were extracted using 90% methanol and processed as described in [43–46] and were kept in −80 °C for further experiments.

Cell cultures

For enzymatic assays, passages 4 and 5, respectively, of normal human epidermal keratinocytes and dermal fibroblasts of the primary cultures established from foreskins following the standards protocols used in our laboratory [36] were used. In addition, we have used immortalized line of adult epidermal melanocytes (PIG-1, gift of Dr. LePoole from Loyola University), which was grown in melanocyte growth media (MGM) supplemented with melanocyte growth factors (MGF) (Lonza Walkersville Inc, Walkersville, MD, USA). Immortalized adult human epidermal keratinocytes (HaCaT) were cultured in Dulbecco's minimal essential media (DMEM) supplemented with 5% charcoal stripped fetal bovine serum (Serum Source International, Inc. Charlotte, NC, USA) and 1% Antibiotic-Antimycotic Solution (Mediatech, Inc. Manassas, VA, USA)[48].

For functional assays, performed at the University of Munster, human epidermal HaCaT keratinocyte line was purchased from ATCC (LGC Standards, Middlesex, UK) while MNT-1 cells representing a human melanotic melanoma line was acquired as a gift from Dr. Cédric Delevoye (Institute Curie, Paris, France). HaCaT cells were cultured in RPMI-1640 supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1% (v/v) streptomycin-penicillin solution [49]. Normal neonatal human melanocytes (Cell Applications, Inc., San Diego, CA, USA) were cultured in Melanocytes Medium254 supplemented with human melanocyte growth supplement (HMGS) (Thermo Fisher Scientific, Waltham, MA, USA). MNT-1 cells were maintained in DMEM medium supplemented with 20% (v/v) heat-inactivated fetal bovine serum, 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 1% (v/v) NEAA, 1% (v/v) streptomycin-penicillin solution at 37 °C in a humidified atmosphere of 5% CO_2 in air [50]. Cells in the logarithmic growth phase were used in all experiments while 80–90% monolayers of confluent MNT-1 cells were harvested with a mixture of 0.05% trypsin-EDTA solution. The media were changed every 48 h.

Cell viability assay

Cells were seeded on 96-well plates at the density of 1.5×10^4 cells/well in the culture medium and thereafter grown to subconfluence. Incubation with NAS and serotonin was performed in dose- (10^{-7}–10^{-3} M) and time-dependent manner (24, 48 and 72 h). Cell viability was evaluated by MTT assay. Briefly, MTT (5 mg/mL) was dissolved in 1×PBS, filtered through a 0.22 μM Millipore® filter and stored at 4 °C. After desired incubation time, cells were washed twice with 1×PBS, then 100 μL of MTT in culture medium (final dilution, 1:10) was added to each well, and incubated for 3 h at 37 °C to allow MTT metabolism. The formazan produced was dissolved in 100 μL acidic isopropanol and absorbance was measured at 595 nm using a BioTek ELx808™ microplate reader (BioTek Instruments, Inc., Winooski, VT, USA).

Measurement of melanin content

Cells were seeded on 6-well plates (Sarstedt, Nümbrecht, Germany) at the density of 3×10^5 cells/well and were allowed to attach overnight. The cells were then incubated for 72 h in fresh medium containing various concentrations of NAS or serotonin dissolved in ethanol as described above. For determination of melanin content, the MNT-1 cells were harvested,

washed with 1×PBS, centrifuged at 1,000×*g* for 10 min (4 °C), and solubilized in 500 μL of 1 N NaOH for 2 h at 80 °C. The absorbance was measured at 405 nm using a BioTek ELx808™ microplate reader, and results were presented as the percentage of the control sample.

DOPA oxidase activity of tyrosinase

MNT-1 cells were seeded on 6-well plates and incubated with NAS or serotonin for 72 h as described earlier. After that, cells were harvested, washed with 1×PBS, centrifuged at 1,000×*g* for 10 min (4 °C), lysed 0.5% Triton® X-100 in 1×PBS on ice. The lysates were subsequently centrifuged at 16,000×*g* for 15 min (4 °C), 300 μL resultant supernatant was added to 300 μL of 5 mM L-DOPA in 1×PBS, and incubated for 1 h at 37 °C. The dopachrome formation was evaluated by measuring absorbance at 475 nm using a BioTek ELx808™ microplate reader, and results were presented as the percentage of the control sample.

Liquid chromatography-mass spectrometry (LC-MS) detection of serotonin and N-acetylserotonin and quantification

In order to detect serotonin and *N*-acetylserotonin, the dried extracts from epidermal and serum samples were re-dissolved in methanol and analyzed by LC-MS as described previously [43–46]. The UPLC (ultra-performance liquid chromatography) separation was performed on a Waters ACQUITY I-Class UPLC system (Waters, Milford, USA) consisting of a binary pump, an autosampler, a column manager, a degasser and a diode-array detector (DAD). An Agilent Zorbax Eclipse Plus C18 column (2.1 × 50 mm, 1.8 μm, Agilent Technologies, Santa Clara, CA, USA) maintained at 35 °C was used with a mobile phase consisting of the following linear gradient of acetonitrile containing 0.1% formic acid: 15% for 1.5 min, 15 – 30% for 0.1 min, 30% for 0.9 min, 30 – 100% for 0.5 min, 100% for 3 min, 100 – 15% for 0.1 min, and 15% for 0.9 min. The flow rate was 0.3 mL/min and the DAD was operated in the range of 200 – 400 nm. The UPLC was connected to a Xevo™ G2-S QTof mass spectrometer (Waters, Milford, USA), a quadrupole (Q) hybrid with orthogonal acceleration time-of-flight (Tof) tandem mass spectrometer (MS) in positive ion mode. The scan range was 50 to 1200 Da in positive mode, and all MS values were collected in centroid mode. The capillary and cone voltages were 3.0 kV and 30 V, respectively. The desolvation gas was maintained at 1000 L/h at a temperature of 500 °C. The cone gas was 100 L/h with a source temperature of 150 °C. The data acquisition rate was 0.3 s, with a 20 second interval. The lockspray frequency was every 20 s using Leucine Enkephalin solution (100 ng/mL) as the lockspray reference compound (*m/z* 556.2771) with a flow rate of 5 μL/min. The MS data were collected with full scan mode with low (6 V) and high (ramp from 20 V to 40 V) collision energy (CE) data channels to get both the parent ions (MS) and the daughter ions (MS/MS). All data were acquired and processed by Waters MassLynx v4.1 software. The relative concentrations of NAS product were calculated from the UPLC Xevo™ G2-S QTof MS peak areas in relation to standards curves generated using the corresponding ions of standards. The values are presented as means ± SE or as individual values.

The limits of detection, accuracy, linearity and recovery have been investigated as described by Carter et al.[51]. The samples were dissolved in methanol and applied LC-MS using Xevo

G2-XS QTof LC-MS system (Waters, Milford, MA, USA). The mass range of 100 to 1,000 Da in positive mode was scanned using the continuum mode with scan time of 1 second. The capillary and cone voltages were 1.7 kV and 40 V, respectively. The desolvation gas flow rate was set on 800 L/h with source temperature of 120 °C. 200 ng/mL leucine enkephalin (m/z = 556.2771) was used as the lockspray reference compound at the flow rate of 10 uL/min with lockspray interval of 10 seconds and scan time of 1 second. UPLC conditions were the same as above except column temperature (25 °C). The mass chromatograms were processed by Waters MassLynx v4.1 software. EIC (Extracted ion chromatograms) were performed using m/z = 160.076± 0.0004 $[M+H\text{-}NH_2COCH_3]^+$ for NAS and 164.077± 0.0004 for NAS-D_7. As an internal standard we have used NAS-D_7 during sample preparation that included a standard curve and recovery determination. For preparation of standard curves we used 0.5, 5, 25 and 500 ng/mL NAS and NAS-D_7 as an internal standard (n = 2).

Supplemental figure 1 shows NAS/NAS-D_7 linearity over a range of 5–500 pg with a slope of 13.75±0.62 and a coefficient of $r^2 \geq 0.9998$. Table 1 shows the intrarun (within run) and interrun (between run) accuracy and precision for the determination. Percent relative error (%RE) represents (Ce−Ct)/Ct] × 100 where Ce is the experimental concentration determined from the calibration curve slope, and Ct represents theoretical concentration. Percent relative standard deviation (%RSD) represents (SD/C_{avg}) × 100, being a measure of assay precision, where C_{avg} is the average concentration calculated, and SD is the standard deviation of C_{avg}. The ratios of NAS/NAS-D_7 were plotted to form calibration curves over concentration rage of 2.28–228 nM of NAS standards (n = 2 for each standard) suspended in fetal bovine serum (FBS). The limits of quantitation (LOQ) were calculated as the ratio of signal to noise and it was 23 for the lowest concentration of NAS, 0.5 ng/mL.

The extraction efficiency was determined as follows, FBS samples containing 0.5, 5, and 50 ng/mL NAS were processed alongside equivalent concentrations of the standard prepared without serum. The NAS-D_7 standard was added to the samples before analysis by LC-MS. The recovery was calculated as the ratio of peak areas, FBS vs. non-FBS. NAS recovery for 0.5, 5 and 50 ng/mL was 157.1, 86.4 and 35.8%, respectively.

Serotonin levels

Serotonin levels in the epidermis and serum were measured using ELISA kit from MyBioSource, Inc. (San Diego, CA, USA) following manufacture's recommendations. Briefly, 100 μL of samples and standard solutions were added to the wells and incubated for 2 h at 37 °C. Biotin-antibody was added to the wells and incubated for 1 h at 37 °C. The wells were washed with wash buffer followed by adding HRP-avidin to each well and incubated for 1 h at 37 °C. After washing with buffer for 5 times, TBM substrate was added to each well and incubated for 20 min at 37 °C. After adding stop solution, the absorbance was measured at 450 nm.

Tryptophan hydroxylase activity assays

Cells were detached and processed as described previously [36]. Cell pellets were suspended in ice cold 5 mM Tris-HCl (pH 7.0) containing 0.2% Triton X-100 and homogenized.

Homogenate was further incubated in an ultrasound bath for 1 min and then used for determination of enzymatic activity. The standard reaction conditions contained 0.1 to 2 mM L-tryptophan, 0.3 mM 6-methyltetrahydropterin, 200 mM ammonium sulfate, 7 mM DTT, 25 μg/mL catalase, 25 μM ferrous ammonium sulfate, 50 mM of 2-*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 7.0), with atmospheric oxygen. The assays was initiated by the addition of 10 μL of a 3 mM stock of 6-MePH4 6-methyltetrahydropterin). The final volume of reaction mixture was 100 μL. The reaction was stopped by addition of 6 M perchloric acid. The production of hydroxytryptophan (TrpOH) was measured using and HPLC with fluorometric detection as described previously [26,30]. The relative concentrations of TrpOH product were calculated from the HPLC peak areas in relation to standards curves generated using the corresponding TrpOH standard. The Km and Vmax values were calculated from Michaelis-Menten plots using Prism 4.00 (GraphPad Software, San Diego, CA, USA).

Immunocytochemical detection of serotonin in human skin

Immunocytochemistry was performed as described previously [18,52]. Briefly, frozen sections were performed on normal human scalp skins from three different donors, obtained after elective plastic surgery with informed consent. The skin was embedded in OCT medium (RA Lamb, East Sussex, UK). Multiple cryosections of different human skins were air-dried, fixed in ice-cold acetone, hydrated in PBS, and blocked in 10% donkey serum. They were incubated with rabbit anti-serotonin polyclonal antibody (abcam ab8882) diluted 1:100 for 1 h. Then the sections were washed in PBS and incubated using the DAKO LSAB2 HRP/3-amino-9-ethylcarbazole (AEC) system (DAKO Ltd, Cabridgeshire, UK) according to the manufacturer's instructions, which includes a biotin-labelled anti-rabbit antibody followed by streptavidin. Negative controls were processed as above, except with primary antibody against serotonin replaced with donkey serum.

Detection of dopamine (DA) and 5HT in cultured skin cells treated with UVB

Detailed description of the experimental design is found in the supplemental file. Briefly human fibroblasts (HF), immortalized HaCaT keratinocytes and co-cultured epidermal keratinocytes and melanocytes were cultured as described previously [53,54]. UVB treatment protocols followed those described by Skoboviat et al [54]. The protocols of cell extraction and electrochemical detection of 5HT after HPLC separation followed the protocols described by Williams et al [55].

Statistics

Student *t*-test was performed as indicated using Prism 4.00 (GraphPad Software, San Diego, CA, USA) presented as means ± SE or SD as indicated. Statistically significant differences are considered when *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ (student *t*-Test) and #, $p<0.05$; ##, $p<0.01$; ###, $p<0.001$ (Mann-Whitney test or One-way ANOVA test).

Results and Discussion

TPH activity in human skin cells

Previously we detected the *TPH1* gene and protein expression in the main cellular compartments of the human and rodent skin including epidermis, dermis and adnexal structures [21,26,30], while the *TPH2* gene was detected predominantly in melanocytes and retinal pigment epithelium cells [19,20]. TPH activity was also detected in rodent melanoma cells using a radiometric method [27], while TrpOH was detected in human melanoma cells by LCMS [56]. In the current study we measured TPH activity in extracts of in human epidermal melanocytes, fibroblasts and keratinocytes by measuring TrpOH production using HPLC with fluorometric detection (Fig. 1). The precise retention time of the product was confirmed by spiking the samples with a Trp(OH) standard. The Michaelis-Menten plots of values obtained after plotting TPH activity vs tryptophan concentrations (0.1 to 2 mM) L-tryptophan showed respective values for Km of 5.08 and 2.83 mM and V max 80.5 and 108.0 μmol/min for melanocytes and fibroblasts. The TPH activity in epidermal keratinocytes was too low to obtain Michaelis-Menten plots. Thus, human dermal fibroblasts and epidermal melanocytes express enzymatically active TPH with low activity detected in epidermal keratinocytes, consistent with previous immunocytochemical studies [18,21,22] and western blot assays [3] performed on the skin or skin cells that showed relatively lower expression of TPH in epidermal keratinocytes in comparison to normal and malignant melanocytes. It is important to say that we do not know how different are levels of TPH activity in skin cells in comparison to pineal gland, since human pineal glands have not been available in our laboratory. However, we expect that these levels will be significantly higher in the pineal gland, since it produces melatonin for systemic use. For comparison in other neuroendocrine system we found that murine pituitary *POMC* gene expression is >1,000 higher in the murine pituitary than murine skin [57] indicating that differences in the expression between skin and central endocrine organs can be substantial.

Characterization and quantification of serotonin and NAS in the epidermis and serum

Previously we demonstrated the ability of the mammalian skin to transform serotonin to NAS and further to melatonin, and measured melatonin metabolism in this organ [26–28,30,47,58]. In the present study we detected serotonin and NAS in the epidermis and human serum using Xevo™ G2 qTof mass spectrometer. As shown in Fig. 2 serotonin was detected in the epidermis at *m/z*=160.1 $[M+H\text{-}NH3]^+$ and 159.1 $[M+H\text{-}H_2O]^+$, while NAS at 219.1 $[M+H]^+$ and 160.1 $[M+H\text{-}NH_2COCH_3]^+$ in extracted-ion chromatogram (EIC) with RT corresponding to serotonin and NAS standards, respectively. Thus, similarly to rodent skin [26–28,30,58], human epidermis is able to produce endogenously NAS. In human serum serotonin was detected at *m/z*=199.1 $[M+Na]^+$ and 177.1 $[M+H]^+$ (Fig. 2B), which was expected. Using the qTOF at extremely precise range 160.076 ± 0.0004 $[M+H\text{-}NH_2COCH_3]^+$ we also detected NAS in the human serum (Fig. 2C).

The histologic distribution of the serotonin antigen was then tested in frozen sections of normal human scalp (Fig. 3). Immunoreactivity was detected in keratinocytes of the basal, suprabasal and spinous layers with strong expression in melanocytes and focal expression in dermal blood vessels (Fig. 3a). Serotonin immunoreactivity was also detected in

keratinocytes of the outer root sheath and hair bulb and follicular melanocytes of the hair follicle and fibroblasts of dermal papilla (Fig. 3b). In the dermis, serotonin immunoreactivity was also present in eccrine glands (Fig. 3c) and mast cells (Fig. 3d). This localization is similar to the previously reported distribution of SNAT, melatonin and TPH antigens in frozen sections of human skin [18,52,59] with one difference: melatonin was predominantly distributed in the upper layers of the epidermis which was not the pattern seen in TPH and serotonin antigen distribution [18,52] (Fig. 3). Thus, we have provided strong evidence that serotonin and NAS are produced in the human epidermis. No secondary antibody binding was observed as evidenced by absence of staining when the primary antibody was replaced by donkey serum (Fig. 3e).

Previously, we have quantified levels of melatonin and its metabolites in the human epidermis [45–47]. In the present studies we measured serotonin levels using ELISA assay and NAS by qTof LC-MS using epidermis obtained from 13 subjects (the same extracts as those used for measurement of melatonin and metabolites [45,46]) and serum. NAS was detected in 13 of 13 serum samples and in 10 of 13 epidermal samples. Supplemental Figure 1 and Table 1 demonstrate the linearity, accuracy and limits of detection of NAS by by qTLC-MS. The detected average values for serum and epidermal samples (2.44 and 43.41 ng/mL, respectively) were within the detection limits and at the linear range of the assay. Since the recovery of NAS at the lower range (0.5 ng/mL) was higher than expected (157.1%), similar to the reported by Carter et al. [51] (123.4%), we submitted an extracted sample of FBS for qTLC-MS analysis using m/z = 160.076 and found a small but detectable corresponding peak in FBS (not shown). This could explain higher than expected recovery of NAS at low range, see above.

As shown in Table 2 serotonin levels in the epidermis were 2.6 times higher than that of NAS, while 121 times higher than NAS in the serum. Epidermal levels of these compounds were independent of race, gender and age. Although we noted higher NAS levels in African-Americans (30 to 50 yrs) vs older Caucasian males (60 to 90 yrs), this difference would require confirmation on a larger cohort of patients (Fig. 4; supplemental Fig. 2). Interestingly, a similar trend was reported for melatonin content in the epidermis [46]. In the human serum there were no differences in serotonin levels depending on age and gender, with slightly higher NAS levels in females than males (supplemental Fig. 2). Backlund et al have recently detected *N*-acetyltryptamine in the rhesus macaque, rat and human plasma samples [60], while Carter et al detected NAS in the serum of two children [51]. The listed by Carter et al [51] values of 15 pg/mL and 24 pg/mL) of plasma levels of NAS in children with attention disorders that were 70 to 100 times lower than reported by us, which requires explanation. These differences can be due to our use of serum samples while the cited authors used plasma or in differences in subjects' selection. We repeated analysis of 13 human serum samples using m/z = 160.076 on Xevo G2-XS QTof LC-MS system and found the values at similar range (6.13 ± 0.67 ng/mL) as in Table 2. Moreover, our cohort was composed of healthy adult individuals while their cohort consisted of two young children's with autism. We also acknowledge the limitation of this study represented by limited number of human subjects (n=13). Therefore, future studies using a large cohort of donors are required to define a precise correlation between race age and health status and NAS levels. It is worthy to cite old literature reporting acetylation of serotonin in the rodent [61] and human

liver [62], which indicates that this organ can supply systemic circulation with NAS. Interestingly, White et al. [62] reported a wide variability in the 13 livers tested to acetylate serotonin.

We also tested the effect of UVB on the production of serotonin in melanocytes, keratinocytes and fibroblasts using a chemiluminescent detection system coupled to HPLC separation (supplemental Fig. 3). Briefly, the basic levels of serotonin in sham irradiated fibroblasts, HaCaT keratinocytes and co-cultured melanocytes and keratinocytes (0 mJ/cm^2) were 120.4, 24.9 and 7.7 pg/10^5 cells, respectively. Only in HaCaT and co-cultured cells did UVB treatment enhance serotonin production at 10 mJ/cm^2. One hundred mJ/cm^2 attenuated this production in all cell types, which may be secondary to the partial toxic effect of this UVB dose [63]. On the other hand UVB inhibited dopamine production in a dose-dependent manner in fibroblasts, HaCaT keratinocytes and melanocytes from the respective basal levels of 26.3, 19.2 and 23.8 pg/10^5 cells (supplemental Fig. 4). Interestingly, catecholamines are produced endogenously by skin cells [64], and dopamine has a potential to inhibit serotonin and melatonin synthesis [24,65–68]. Therefore, our preliminary data are encouraging for further studies on UVB regulation of interactions between serotoninergic/melatoninergic and dopaminergic systems.

Serotonin's role in the regulation of pigmentary responses is complex and dependent on ligand activation of a particular receptor [8,41,69,70]. In melanophores pigment granule dispersion or aggregation is dependent on activation on 5HT3 or 5HT1/2, respectively [69]. In human melanomas serotonin or serotonin uptake inhibitors were reported to inhibit melanogenesis [8,70]. However, 5HT1A/2A or 5HT2B can stimulate or inhibit melanogenesis, respectively [40,41]. Therefore, we tested the phenotypic effects of serotonin and NAS on melanogenic activity using the pigmented melanoma line MNT-1 (Fig. 5). Thus, melanin synthesis was significantly inhibited only by high doses (10^{-4} and 10^{-3} M) of serotonin and NAS with respective IC_{50} of 2.0×10^{-4} and 5.2×10^{-5} M (Fig. 5A, B). The need for high serotonin concentration suggests that anti-melanogenic effect is independent of membrane bound 5TH receptors. A similar pattern of regulation was observed for tyrosinase activity with corresponding IC_{50} for serotonin and NAS being 2.6×10^{-4} and 1.9×10^{-4} M (Fig. 5C, D). Although the described effects of serotonin are in agreement with studies on human melanomas [8,70] and human melanocytes and murine immortalized melanocytes [41] we believe that the effect will be dependent on cellular genotype and composition of the corresponding receptors. As it relates to NAS, this is the first demonstration of an inhibitory effect on melanogenesis, since previous studies on inhibition of melanogenesis by 10^{-4} M NAS showed a lack of effect in rodent melanomas [71] or some inhibition in anagen murine skin but without statistical significance [72]. These combined, and presented results and literature reports demonstrate that the regulation of melanin pigmentation by serotonin and NAS is complex, dependent on species, normal and malignant phenotype, and receptor expression and a probability involve metabolic transformation 5HT→NAS→melatonin, since human melanomas can transform tryptophan to melatonin [56] and MNT-1 expresses *TPH1* and *HIOMT* [73].

We also tested the effect of serotonin and NAS on growth of MNT-1 melanoma and normal epidermal melanocytes in cell culture (supplemental Fig. 5; Fig. 6). While NAS had no

marked effect on melanoma cell growth, serotonin inhibited it only at very high concentration of 10^{-3} M (supplemental Fig. 5). In the case of normal epidermal melanocytes serotonin and NAS moderately inhibited cell growth with respective IC_{50} of 2.8–6.4 × 10^{-6} and 0.3–2.6 × 10^{-4} M (Fig. 6). The inhibitory effect of serotonin on melanocytes growth is consistent with previous report [26]. These results show that while serotonin and to lower degree NAS can inhibit growth of human melanocytes, while melanoma cells are becoming resistant to such regulation, especially in case of NAS. Although the above concentrations of the ligands are unphysiological as relates to the serum levels, they are produced locally and, therefore, are considered as a part of physiological local response system. Regarding NAS, it is noteworthy that it can protect human keratinocytes and melanocytes against UVB induced damage [42,74]. It has a similar effect on induction of genes involved in anti-oxidative response, attenuation of ROS and DNA damage and increases nuclear levels of p53 phosphorylated at Serine 15, similarly to the effects excreted melatonin and its metabolites [42,74]. The above results suggest that NAS is not a mere product of serotonin or melatonin metabolism but it has its own biological activity that includes regulation of pigment cells and involvement in photoprotective activities. The relatively high concentration of NAS necessary to achieve the phenotypic effects suggests an existence of a nuclear receptor for this ligand.

Conclusions

In this report we provide evidence that serotonin and NAS are produced endogenously in the epidermal, dermal and adnexal compartments of human skin and in cultured skin cells that also show detectable tryptophan hydroxylase activity, a rate-limiting enzyme in the serotoninergic pathway. Epidermal serotonin levels were independent of race, gender and age, but its production could be affected by environmental factors such as UVB. NAS epidermal levels were higher in young African-Americans vs old Caucasian males having a similar trend to that reported previously for melatonin. The present and reported previously detection of NAS in human serum indicated its availability to serve as a substrate for enzymatic transformation to melatonin in peripheral organs expressing HIOMT. Therefore, NAS can enter the circulation from different peripheral organs expressing NAT1/2, especially liver and perhaps skin. Nevertheless, its presence in the circulation and peripheral organs could explain why the C57BL6 mouse with inactive AANAT can produce melatonin. Serotonin and NAS can regulate the skin pigmentary activity including an antiproliferative effect in normal melanocytes and an inhibition of melanogenesis in melanoma cells. This suggests that NAS is not merely an intermediate or product of serotonin and melatonin metabolism but that it also has bioregulatory activity in skin cells, in addition to previously reported protection against the UVB induced damage. This property is shared by melatonin and its metabolites. In conclusion, we propose that cutaneous production of serotonin and NAS is a part of a local neuroendocrine system that regulates skin homeostasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Abbreviations:

NAS	*N*-acetylserotonin
yrs	years old
UVB	ultraviolet B
FBS	fetal bovine serum
TCA	trichloroacetic acid
SDS	sodium dodecyl sulfate
ESI	electrospray ionization
MS	mass spectrometry
LC-MS	liquid chromatography mass spectrometry
HPLC	high-pressure liquid chromatography
UPLC	ultra-performance liquid chromatography
qTOF	quadrupole time-of-flight
NAS-D_7	*N*-acetylserotonin-d_7

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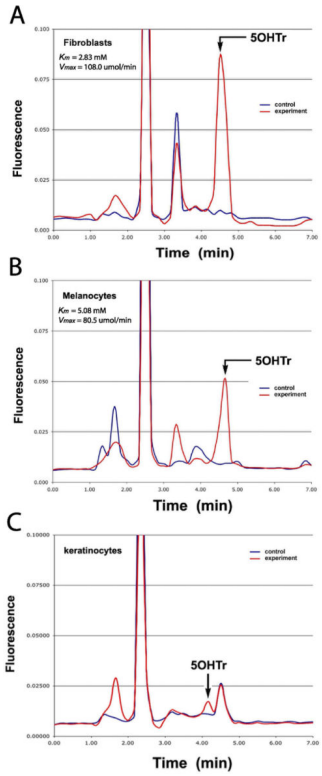

Figure 1.

Tryptophan hydroxylase (TPH) activity in skin cells. TPA activity was measured as production of 5-hydroxytryptophan (5OHTr) in extracts from dermal fibroblasts (passages 4 and 5) (A),immortalized line of epidermal melanocytes (PIG-1) (B) and keratinocytes (passages 4 and 5)(C) using HPLC with fluorimetric detection. Experimental represents samples incubated with L-tryptophan, while control samples were incubated without the substrate. The assays were repeated 4 times.

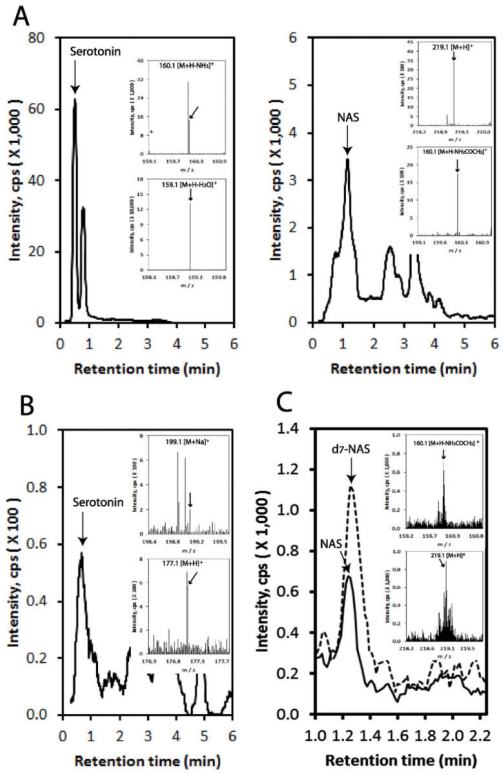

Figure 2.

Detection of serotonin and NAS in human epidermis and serum. LC-MS was performed as described in *Materials* and *Methods*. EICs are shown using *m/z* = 159.1 $[M+H\text{-}H_2O]^+$ (serotonin in epidermis), 219.1 $[M+H]^+$ (NAS in epidermis), 199.1 $[M+Na]^+$ (serotonin in serum) and 160.076 $[M+H\text{-}NH_2COCH_3]^+$ (NAS in serum). Pre-purified sample (using a long column (WatersC18 column, 250 × 4.6 mm, 5 μm particle size) with a gradient of acetonitrile in water (40–100%) at a flow rate of 0.5 ml/min (15 min), followed by a wash with 100% acetonitrile for 30 min at a flow rate of 0.5 ml/min and for 20 min at a flow rate of 1.5 ml/min) was used for serotonin in epidermis. All other EICs were performed using crude extracts. A, detection of serotonin and NAS in the epidermis; B, detection of serotonin in the serum. C, detection of NA in the serum using Xevo G2-XS QTof LC-MS system (Waters, Milford, MA, USA). The analysis was described in Materials and Methods. NAS-D_7 was used as an internal standard (broken line), and EIC was performed using *m/z* = 160.076 ± 0.0004 for Abs Window (Da) for NAS and using *m/z* = 164.077 for NAS-D_7. Inserts show mass spectra of RTs corresponding standards. Thirteen independent assays were performed for different samples with two LC/MS repeats for each sample. The epidermal extracts were obtained from intact human skin and serum was collected as described in Materials and Methods.

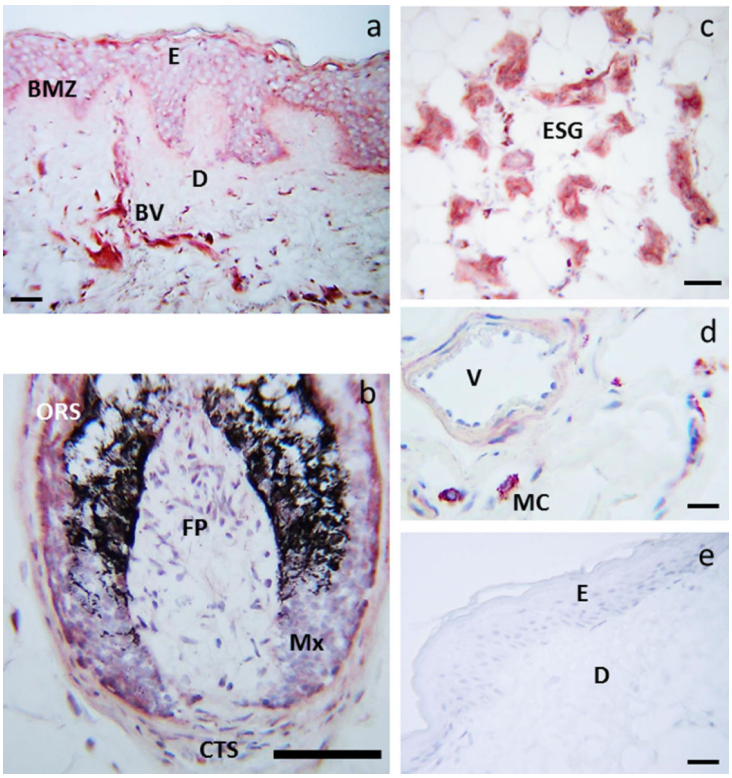

Figure 3.

Immunolocalization of serotonin in human scalp. Serotonin immunoreactivity was detected cytoplasmically and membranously in keratinocytes and melanocytes of the basal layer, and in keratinocytes of the suprabasal and spinous layers, as well as in dermal blood vessels (BV) (a), ORS and matrix cells of the hair follicle, FP and CTS (b), ESG (c) and mast cells (d). Control: primary antibody replaced with donkey serum and secondary antibody only (e). BMZ: basement membrane zone, CTS: D: dermis, E: epidermis, ESG: eccrine/sweat glands, FP: dermal papilla fibroblasts, Mx: hair follicle matrix, ORS: outer rout sheath, V: lumen of the blood vessel. Scale bar: 20 μm (a), 100 μm (b), 50 μm (c), 13 μm (d), 23 μm (e). The immunostains are from the scalp skin of the 35 years old male donor, a representative of three donors with multiple stains performed for each skin sample

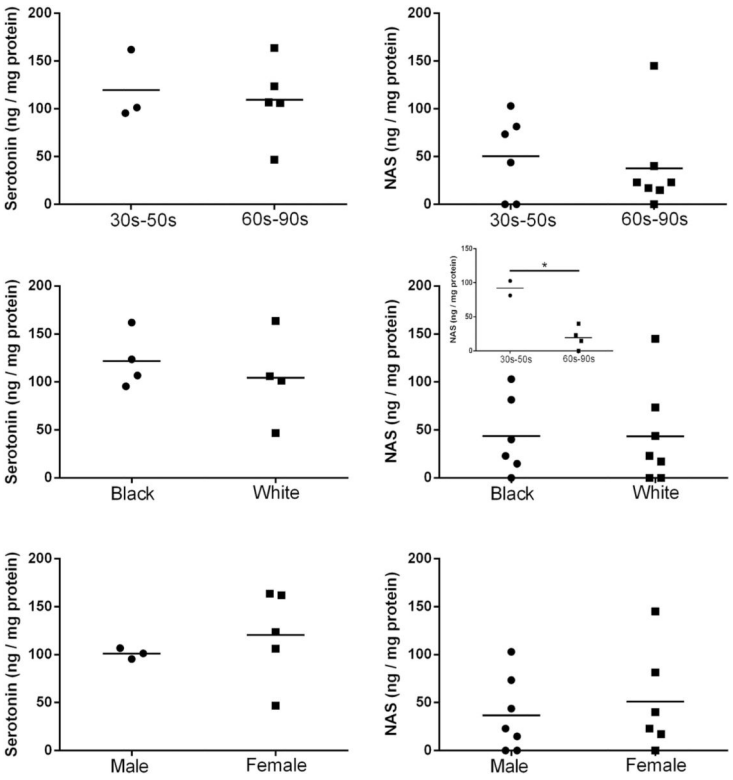

Figure 4.

Levels of serotonin and NAS in the epidermis. Serotonin was quantified using ELISA kit from MyBioSource, Inc. (San Diego, CA) and NAS was quantified with qTOF LC-MS (EIC) using corresponding standard (m/z = 219.1 $[M+H]^+$, *p < 0.05 at Student t-test.

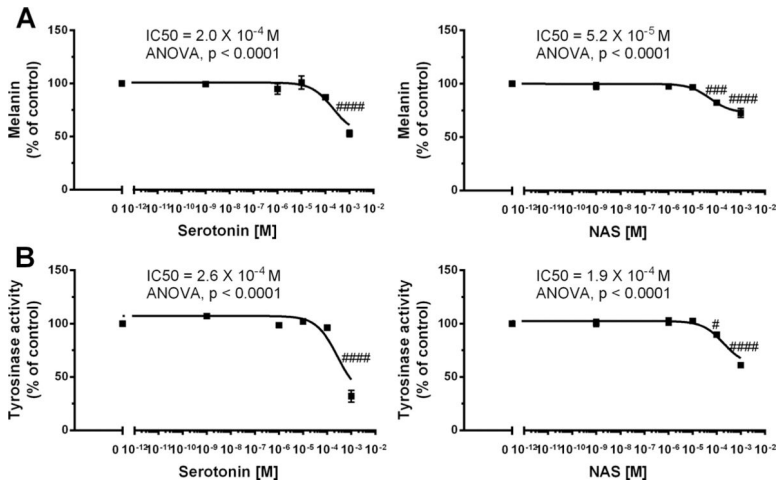

Figure 5.

Inhibition of melanogenesis by serotonin and NAS in MNT-1 melanoma cells Cells were incubated with graded concentrations of serotonin or NAS for 72 h in a dose-dependent manner and proceeded for melanin assay (A) and tyrosinase activity (B) as described in *Materials* and *Methods*. Data were presented as the mean±SE (n=6), while values were normalized and expressed as percentage of the control value i.e. cells incubated in presence of 0.2% ethanol. Statistically significant differences were indicated as $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$, $^{\#\#\#\#}P < 0.0001$ by one way ANOVA. The experiments were independently repeated three times with similar results.

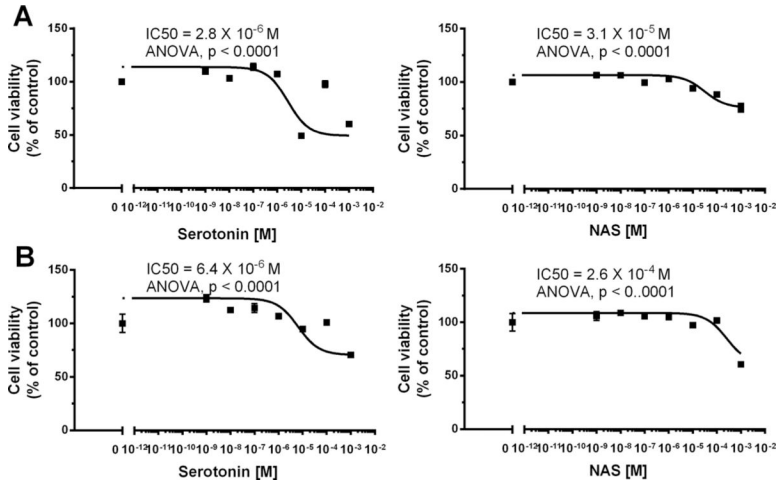

Figure 6.

Inhibition of normal neonatal epidermal melanocytes growth by serotonin and NAS. Dose dependent curves of MTT viability assay were generated after 48 (A) and 72 (B) h of treatment with serotonin and NAS. Data were presented as the mean±SE (n=6), while values were normalized and expressed as percentage of the control value i.e. cells incubated in presence of 0.2% ethanol. The neonatal keratinocytes were from the commercial sources and were grown as described in the materials and methods. The experiments were independently repeated three times with similar results.

Table 1.

Precision and accuracy of the NAS assays

ng/mL	Intrarun				Interrun (9 days)	
	1-st		2-nd			
	RE (%)*	RSD (%)**	RE (%)*	RSD (%)**	RE (%)*	RSD (%)**
0.5	28.87	2.61	12.59	129	19.54	8.22
5	−7.78	5.01	15.24	8.00	5.36	13.25
50	−4.94	4.82	16.78	1.35	7.54	12.15

*, Relative error: $[C_e-C_t)/C_t]$ X 100 where C_e is the experimental concentration and C_t is theoretical concentration;

**, Percent relative standard deviation: (SD/C_{avg}) X 100 where C_{avg} is the average concentration and SD is the standard deviation

Table 2.

Serotonin and NAS content in the human epidermis and serum

	Serotonin	NAS
Epidermis (ng/mg protein)	113.18 ± 13.34 (n=13)	43.41 ± 12.45 (n=13)
Serum (ng/mL serum)	295.33 ± 17.17 (n=8)	2.44 ± 0.45 (n=13)

The data represent averages ±SE from different patients (n) with the assay repeated at least 3 times.