Serotonin activates murine alveolar macrophages through 5-HT$_{2C}$ receptors

Zbigniew Mikulski,1 Zbigniew Zasłona,2* Lidija Cakarova,3 Petra Hartmann,1 Jochen Wilhelm,3 Laurence H. Tecott,4 Juergen Lohmeyer,2 and Wolfgang Kummer1

1Institute of Anatomy and Cell Biology, 2Department of Internal Medicine, and 3Department of Pathology, Universities of Giessen and Marburg Lung Center, Excellence Cluster Cardio-Pulmonary System, Giessen, Germany; and 4Department of Psychiatry, University of California, San Francisco, California

Submitted 28 January 2010; accepted in final form 21 May 2010

Mikulski Z, Zasłona Z, Cakarova L, Hartmann P, Wilhelm J, Tecott LH, Lohmeyer J, Kummer W. Serotonin activates murine alveolar macrophages through 5-HT$_{2C}$ receptors. Am J Physiol Lung Cell Mol Physiol 299: L272–L280, 2010. First published May 21, 2010; doi:10.1152/ajplung.00032.2010.—Serotonin (5-HT), known as neuromodulator, regulates immune responses and inflammatory cascades. The expression and function of 5-HT receptors on alveolar macrophages (AM), which are the major fraction of pulmonary immune cells, remain elusive. Therefore, we determined the expression of 5-HT type 2 receptors and investigated the effects evoked by stimulation with 5-HT in AM compared with alveolar epithelial cells (AEC). Quantitative PCR (qPCR) analysis revealed expression of the receptors 5-HT$_2A$, and 5-HT$_2B$ in AEC and of 5-HT$_{2C}$ in AM. In AM, 5-HT (10$^{-5}$ M) induced a rise in intracellular calcium concentration ([Ca$^{2+}$]i) that was initiated by release of Ca$^{2+}$ from intracellular stores and depended on extracellular Ca$^{2+}$ in a sustained phase. This 5-HT-induced increase in [Ca$^{2+}$]i was not observed in AM treated with the 5-HT$_{2C}$-selective inhibitor RS-102221 and in AM derived from 5-HT$_{2C}$-deficient mice. AM stimulated with 5-HT (10$^{-5}$ M) showed increased expression of CCL2 (MCP-1) mRNA as assayed by qPCR at 4 h and augmented production of CCL2 protein as determined by dot-blot assay and ELISA at 24 h. Notably, in 5-HT$_{2C}$-deficient AM, CCL2 production was not induced by 5-HT treatment. Moreover, transcriptional responses to 5-HT exposure assayed by microarrays were only observed in AM from wild-type animals and not in AM derived from 5-HT$_{2C}$-deficient mice. Taken together, these data demonstrate the presence of functional 5-HT$_{2C}$ receptors on AM and suggest a role of 5-HT as novel modulator of AM function. These effects are exclusively driven by the 5-HT$_{2C}$ receptor, thereby providing the potential for selective intervention.

lung macrophage; serotonin receptor; CCL2

SEROTONIN (5-hydroxytryptamine; 5-HT) is well-known for its role in vasoconstriction as well as in diverse biological processes such as tissue regeneration (28), platelet activation (8), and immune system regulation (36). Outside the central nervous system, most of the body’s 5-HT is produced by enterochromaffin cells in the gut and taken up by and stored in chromaffin cells in the gut and taken up by and stored in vascular wall injury, LPS (45), or allergen challenge (51). Together with 5-HT released from mast cells (25), this results in increased 5-HT levels in inflammation (36). In addition, activated T cells (40) and macrophages (38) express tryptophan hydroxylase, the rate-limiting enzyme for 5-HT biogenesis. Dendritic cells (40), monocytes (14), and macrophages (44) express SERT, which translocates 5-HT across the plasma membrane.

Immune cells also respond to 5-HT. Serotonin receptors build up a large family including ionotropic cation channels (5-HT$_3$) and six types of metabotropic G protein-coupled receptors (20). Various subtypes of metabotropic 5-HT receptors have been identified on B cells, T cells, dendritic cells, peripheral blood mononuclear leukocytes, and macrophages (6, 10, 16, 21, 30, 35, 36). In inflammatory states in general, 5-HT regulates cell growth and division, cytokine synthesis, and chemotaxis (1, 24, 37, 51). In the lung in particular, 5-HT$_2$ receptors have been implicated in the pathogenesis of pulmonary fibrosis (13) and pulmonary hypertension (19). The 5-HT$_2$ receptor class includes 5-HT$_{2A}$, 5-HT$_{2B}$, and 5-HT$_{2C}$ receptors, which are coupled to G$_{a/11}$ proteins. Receptor activation triggers signaling cascades resulting in formation of inositol phosphates and intracellular calcium elevation (26), an intracellular signal known to initiate an interferon response and cytokine release (48, 54).

Alveolar macrophages (AM) initiate and orchestrate immune responses by pathogen sensing as well as cytokine and chemokine release. They possess surface receptor sites capable of binding physiological concentrations of 5-HT (12). The acute effects of 5-HT on freshly isolated AM and their in vivo responses to stimulation with 5-HT are unknown. Thus we examined the expression profiles of 5-HT$_3$ receptors in mouse AM compared with that of alveolar epithelial cells (AEC) and determined AM responses to stimulation with 5-HT. We show that AM express functional 5-HT$_{2C}$ receptors, which are responsible for intracellular calcium concentration ([Ca$^{2+}$]i) rise, transcriptional regulation, and modulation of chemokine production on 5-HT stimulation.

MATERIALS AND METHODS

Animals. Wild-type C57BL/6N mice were purchased from Charles River (Sulzfeld, Germany). The generation of animals deficient for the 5-HT$_{2C}$ receptor was described previously (50). Mice were bred by mdf Diagnostics (Wendelsheim, Germany) using heterozygotes as breeders, and littermate wild-type mice resulting from this colony were used as controls in experiments involving 5-HT$_{2C}$ receptor knockout mice. All animals were kept with free access to food and water. Mice were used throughout the study between 8 and 12 wk of age. Experimental protocols involving animals were approved by institutional and local government authorities, following the current version of the German Law on the Protection of Animals as well as the National Institutes of Health principles of laboratory animal care.

Isolation and culture of mouse AM. Bronchoalveolar lavage (BAL) fluid was obtained by cannulating the trachea with a shortened 21-gauge needle attached to a 1-ml insulin syringe, followed by repeated intratracheal instillations with 500-µl aliquots of sterile...
PBS/2 mM EDTA (pH 7.2) until a BAL fluid volume of 5 ml was recovered, according to previously described protocols (32). Subsequently, BAL fluid was centrifuged at 400 g for 10 min at 4°C. After washing with PBS, AM were resuspended in DMEM/F-12 GlutaMax-I medium (Invitrogen, Karlsruhe, Germany) for Ca²⁺ measurements and in RPMI 1640 (PAA, Pasching, Austria) for all other experiments. Both media were supplemented with penicillin (100 U/ml) and streptomycin (0.1 mg/ml; PAA). Before each experiment, the composition and integrity of BAL cells was monitored by microscopy, and cells were purified by adherence for ≥1 h and washed. Purified AM were cultured in RPMI 1640 medium containing 1% BSA (for dot-blot assay) or 1% dialyzed FCS (for ELISA experiments; PAA), L-glutamine, and penicillin/streptomycin at 5 × 10⁵ cells/well in a 24-well tissue culture plate for dot-blot array and at 2.5 × 10⁵ cells/well in a 48-well plate for ELISA measurement. For quantitative PCR (qPCR) and microarray, AM were stimulated with 5-HT (10⁻⁵ M; Sigma-Aldrich, Taufkirchen, Germany) for 4 h. For dot-blot assay, 10⁻⁵ M, and for ELISA, 10⁻⁵, 10⁻⁷, or 10⁻⁹ M 5-HT concentration was used for 24 h.

**Isolation of CD11c⁺ interstitial macrophages.** Interstitial macrophages (IM) from lungs were isolated according to the modified protocol described previously (52). Briefly, lavaged lungs were perfused with 20 ml of sterile HBSS until free of blood by visual inspection and then removed and transferred into petri dishes containing 0.7 mg/ml collagenase A (Roche, Penzberg, Germany) and 50 μg/ml DNase I (Serva, Heidelberg, Germany) in RPMI 1640 medium. Lungs were minced and cut into small pieces, agitated on a shaker for 30 min at room temperature, and then incubated at 37°C for 30 min in a humidified atmosphere containing 5% CO₂. Cell aggregates were dispersed by repeated passage through a syringe and filtered through a 200- and a 40-μm cell strainer (BD Biosciences, Heidelberg, Germany) to obtain single cell suspension. Subsequently, cells were rinsed with HBSS and PBS/2 mM EDTA/0.5% FCS (PAA), followed by incubation with an excess concentration of unspecific IgG (Octagam; Octapharma, Springe, Germany) to reduce nonspecific antibody binding. After washing with PBS/2 mM EDTA/0.5% FCS, cells were incubated with magnetic bead-conjugated anti-CD11c antibodies (Miltenyi Biotec, Bergisch Gladbach, Germany) followed by magnetic separation according to the manufacturer’s instructions. Subsequently, the cell population containing CD11c⁺-positive cells was stained with CD11c antibody conjugated to phycoerythrin (clone HL3; BD Pharmingen, Heidelberg, Germany). Cell sorting was performed with a FACSVantage SE flow cytometer equipped with a DiVA sort option and an argon-ion laser at 488-nm excitation wavelength (λ). Cells positive for CD11c and with high autofluorescence in FL1 channel were identified as IM as described previously (57) and outlined in Fig. 1A. To keep isolation procedure as similar as possible
for cells used for the receptor expression experiment, AM obtained by BAL were also FACS-sorted as described previously (57).

Isolation and culture of AEC. Type II AEC were isolated by the method developed by Corti et al. (7), with some modifications as specified elsewhere (18, 31). In brief, mice were sedated with isoflurane (Abbott, Wiesbaden, Germany), and lungs were perfused with 20 ml of sterile HBSS. After enzymatic digestion, single cell suspension was centrifuged, resuspended, and incubated with biotinylated rat anti-mouse CD45 (4.5 μg/ml × 107 cells), CD16/32 (3.4 μg/ml × 107 cells), and CD31 (2 μg/ml × 107 cells; all antibodies from BD Pharmingen) for 30 min to deplete leukocytes and endothelial cells. The contaminating cell types were removed by incubation with streptavidin-linked magnetic particles (Dynabeads; Invitrogen) and subsequent magnetic separation with MACS magnetic separator (Miltenyi Biotec). The supernatant was recovered, and the purity of the AEC II preparation was routinely assessed by pro-SF-C staining (1:800; Chemicon International, Schwalbach, Germany) with flow cytometry (4). The final cell suspensions always consisted of >95% AEC. Viability was always >95%, as assessed by trypan blue dye exclusion. For qPCR, 2.5–5.0 × 105 AEC/well were seeded in a 24-well cell culture plate and cultured for 3 days. AEC were kept in DMEM (high glucose; 4 g/l) containing penicillin (100 U/ml), streptomycin (0.1 mg/ml), 25 mM HEPES buffer (PAA), and 10% FCS for the first 16–24 h and then starved in medium supplemented with 0.1% FCS.

RNA isolation. RNA from cell populations was isolated using an RNeasy Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Quantification and purity of RNA was determined with an Agilent Bioanalyzer 2100 (Agilent Biosystems, Waldbronn, Germany). Only those RNA preparations exceeding absorbance ratios of A260/280 1.90 and with a total amount of RNA 1.5 μg were used for microarray experiments. The cDNA synthesis, use of reagents, and incubation steps were performed as described previously (57).

Microarray. Purified total RNA was amplified and fluorescently-labeled using the dual-color QuickAmp kit (Agilent Technologies, Palo Alto, CA) following the kit instructions. The samples were labeled with either Cy3 or Cy5 to match a balanced dye-swap design. Cy3- and Cy5-labeled RNA were hybridized overnight to 44K 60-mer oligonucleotide-spotted microarray slides (Mouse Whole Genome 44K, Agilent Technologies). RNA from 5-HT-stimulated AM was hybridized against RNA from corresponding control AM. Four hybridizations each were performed for wild-type and for 5-HT2C knockout mice, respectively. Hybridization and subsequent washing and drying of the slides were performed following the Agilent hybridization protocol. The dried slides were scanned using the GenePix 4100A scanner (Axon Instruments, Downingtown, PA). Photomultiplier gains were set to match the intensity histograms of both channels. Image analysis was performed with GenePix Pro 5.0 software, and calculated values for all spots were saved as GenePix results files. Stored data were evaluated using the R software (49) and the limma package from BioConductor (http://www.bioconductor.org) (17). Data from duplicate spots were averaged before further analysis. The M/A data were LOESS normalized (47) before averaging. Genes were ranked for differential expression using a moderated t-statistic (46). Candidate lists were created by adjusting the false-discovery rate to 5%. The complete data set is accessible online in the GEO database (http://www.ncbi.nlm.nih.gov/geo) under the accession number GSE19214.

PCR and microarray validation. To measure the expression of 5-HT2 receptors in AM, IM, and AEC and to validate the results obtained by microarray, RNA transcripts of selected genes were analyzed on independently prepared samples by qPCR. Three and four biological replicates were used for the receptor expression and microarray result validation, respectively. Data from two technical replicates were averaged and analyzed using the change in cycle threshold (∆Ct) method for the calculation of relative changes in gene expression (29), with the formula ∆Ct = Ctref = Cttarget where Ctref and Cttarget are the Ct values of the reference gene (hydroxymethylbilane synthase; hmb2) and the target gene, respectively. Fold change and ∆∆Ct were calculated according to Livak and Schmittgen (29). The qPCR analysis was performed with a Sequence Detection System 7900 (PE Applied Biosystems, Darmstadt, Germany). Reactions (final volume: 25 μl) were set up with 13 μl of SYBR Green PCR Core Reagents (Invitrogen), 1 μl of 25 mM MgCl2, 5 μl of cDNA sample, 0.5 μl of each forward (f) and reverse (r) primers (5-pmol final concentration) and 5 μl of H2O. Melting curve analysis revealed purity of the products. For RT-PCR reactions, 2.5 μl of buffer II (100 mM Tris-HCl, 500 mM KCl, pH 8.3), 2 μl of 15 mM MgCl2, 0.6 μl of dNTP (10 mM each), 0.5 μl of each primer (5 pmol), and 0.125 μl of AmpliTaq Gold polymerase (5 U/μl; Applied Biosystems, Foster City, CA) were added to 5 μl of cDNA and made up to a final volume of 25 μl with H2O. Cycling conditions were 10 min at 95°C, 40 cycles with 20 s at 94°C, 20 s at 60°C, 20 s at 72°C, and a final extension at 72°C for 7 min. PCR products were separated by electrophoresis on a 2% agarose gel in Tris-acetate-EDTA buffer. The following intron-spanning primer sequences were used: hmb2, 5'-TGG TGA AGG TCG GTG TGA AC-3' (f), 5'-TGA ATT TGC GGT CAG AG-3' (r); htr2a, 5'-ATA GCC GCT TCA ACT CCA GA-3' (f), 5'-TCA TTC TGT AGC CCG AAC AC-3' (r); htr2b, 5'-GGG CTA GTG CAT TCA TCA AGA-3' (f), 5'-CCT ACA GGT GAC ATT

Fig. 2. 5-HT induces Ca2+ release from intracellular stores followed by extracellular Ca2+ influx. Measurements of intracellular calcium concentration ([Ca2+]i) in AM were performed by the fura 2 method. A: 5-HT (10-5 M) induced a fast rise in [Ca2+]i, with a subsequent short decline and a longer lasting phase of elevated [Ca2+]i. In AM stimulated with ATP (10-4 M), the Ca2+ response was weaker compared with 5-HT. B: dependency of the 5-HT-induced [Ca2+]i rise on extracellular Ca2+. Cells exposed to 5-HT (10-5 M) in Ca2+-containing buffer showed sustained increase in [Ca2+]i, whereas cells stimulated in buffer without Ca2+ exhibit only a transient rise. Number of measured cells is given in parentheses. ***p < 0.001.
was not blunted by the drug.

Measurements of \( [\text{Ca}^{2+}]_i \) were stimulated with 5-HT (10^{-5} M) and ATP (10^{-4} M). Response to the 5-HT stimulus was blocked in treated cells, whereas the reaction to ATP

\[
\text{TG-3} \text{CTT ATG TAC TGA GG-3} (\text{f}), \text{5'=GCT TCT TCA TGT GAG AGG TG-3} (\text{r}); \text{nt5e, 5'-CAG CCT GAA GAC TGT G3-3} (\text{f}), \text{5'-TCC AGG GCT TTC GTG TAA TA-3} (\text{r}); \text{mvo1d, 5'-ATG AGC TGA AAC GCA AGG AC-3} (\text{f}), \text{5'-GTT GAC GGT CAG TGA CAA AA-3} (\text{r}); \text{ccl2 5'-AGC ATC CAC GTG TTG GCT C-3} (\text{f}),
\]

were performed after 1.5–6 h in primary culture. For each of the experiments presented in Fig. 2A and B, and Fig. 3A, \( n = 3 \) animals were used (total \( n = 9 \)), whereas data presented in Fig. 3B were generated from \( n = 8 \) animals (4 5-HT2c receptor-deficient animals and 4 littermate controls). Measurements were done in HEPES buffer containing 5.6 mM KCl, 136.4 mM NaCl, 1 mM MgCl2, 2.2 mM CaCl2, 11 mM D-glucose, and 10 mM HEPES. In some experiments, CaCl2 was omitted from the buffer composition. Cells were loaded for 30 min with 3.3 \( \mu \)M fura 2-acetoxymethyl ester (Invitrogen) and washed 3 \times 10 \text{ min}. Fura 2 was excited at 340- and 380-nm \( \lambda \), and fluorescence was collected at \( \lambda > 420 \text{ nm} \). The fluorescence intensity ratio of 340/380 nm was recorded. Cells were exposed to 5-HT alone (10^{-8}, 10^{-5} M) or in the presence of the 5-HT2c blocker RS-102221 (10^{-4} M) and ATP (10^{-4} M) serving as a positive control. Cells that responded to neither 5-HT nor ATP by \( \geq 5\% \) change in \([\text{Ca}^{2+}]_i\), were excluded from further analysis. Viability of the cells was monitored after measurements with trypan blue exclusion. Ratio values were normalized to 100\% at the beginning of recording. Data are shown as means \( \pm \text{SE} \).

**Dot-blot-based mouse cytokine antibody array.** Cells were stimulated with 10^{-5} M 5-HT for 24 h. In controls, 5-HT was omitted from media composition. Culture supernatants were used for qualitative measurement of cytokine expression using the Mouse Cytokine Antibody Array, Panel A (ARY006), as recommended by the manufacturer (R&D Systems, Wiesbaden, Germany).

**ELISA.** The quantification of murine CCL2 (MCP-1) protein in culture supernatants was performed by a commercially available ELISA kit following the instructions of the manufacturer (R&D Systems, Minneapolis, MN). Coefficients of variation for ELISA experiments were \( < 15\% \).

**Statistical analysis.** Data from qPCR 5-HT receptor analysis were analyzed by ANOVA with Tukey post hoc test for multiple comparisons. Data from \([\text{Ca}^{2+}]_i\), measurements did not follow a normal distribution and were analyzed by the nonparametric ranked-based Mann-Whitney test. Correlation between coefficient values from microarray experiment and \( \Delta\text{Ct} \) values from qPCR of selected genes was measured with Pearson product-moment correlation.
coefficient as one-sided test. Data from ELISA and qPCR of selected genes were analyzed with Student’s t-test. Tests were performed using SPSS software (SPSS Software, Munich, Germany). Throughout, \( P \leq 0.05 \) was considered significant, and \( P \leq 0.01 \) as highly significant.

RESULTS

AM and AEC differentially express type 2 serotonin receptors. The expression of 5-HT receptors in lung macrophages isolated by high-purity cell sorting, and primary isolated AEC was analyzed by qPCR. Alveolar and interstitial macrophages express high levels of mRNA coding for 5-HT\(_{2C}\) and lower quantities of 5-HT\(_{2A}\) and 5-HT\(_{2B}\) receptor mRNAs. The latter 2 are expressed by AEC (5-HT\(_{2A}\): 115- and 73-fold, 5-HT\(_{2B}\): 32- and 15-fold expression of AM and IM, respectively), which do not express detectable mRNA levels of the 5-HT\(_{2C}\) receptor (Fig. 1B).

5-HT potently stimulates [Ca\(^{2+}\)]\(_i\) rise in AM. In freshly isolated mouse AM, 5-HT (10\(^{-8}\), 10\(^{-5}\) M) induced a rapid rise in [Ca\(^{2+}\)]\(_i\) followed by a brief decrease and a subsequent long-lasting phase of increased [Ca\(^{2+}\)]\(_i\). The amplitude of 5-HT-induced [Ca\(^{2+}\)]\(_i\) rise was enhanced by 39% in cells treated with 10\(^{-5}\) compared with 10\(^{-8}\) M 5-HT (\( P < 0.0001 \)). The Ca\(^{2+}\) response induced by 5-HT (10\(^{-5}\) M) was compared with the stimulation with ATP (10\(^{-4}\) M), an agonist of P2Y receptors expressed by AM (3). Treatment with ATP evoked a smaller and shorter rise in [Ca\(^{2+}\)]\(_i\), (Fig. 2A). To examine the dependence on extracellular Ca\(^{2+}\) of this rise in [Ca\(^{2+}\)]\(_i\), AM were exposed to 5-HT in Ca\(^{2+}\)-free buffer. Under these conditions, AM exhibited a transient rise in [Ca\(^{2+}\)]\(_i\), whereas the phase of long-lasting elevated [Ca\(^{2+}\)]\(_i\) was lost (Fig. 2B).

The 5-HT-induced rise in [Ca\(^{2+}\)]\(_i\) is mediated by the 5-HT\(_{2C}\) receptor. To test whether the 5-HT-induced increase in [Ca\(^{2+}\)]\(_i\) is mediated by the 5-HT\(_{2C}\) receptor, AM were incubated with the selective 5-HT\(_{2C}\) blocker RS-102221 (10\(^{-8}\) M) 2 min before stimulation with 5-HT. This pretreatment markedly inhibited the 5-HT-induced rise in [Ca\(^{2+}\)]\(_i\), without affecting the ATP stimulatory effect (Fig. 3A).

Accordingly, 5-HT had no effect on [Ca\(^{2+}\)]\(_i\) in AM from 5-HT\(_{2C}\) receptor knockout mice, whereas cells from littermate wild-type animals responded with a rapid rise in [Ca\(^{2+}\)]\(_i\). Cells from both mouse strains were normally reactive to ATP, which served as a positive control (Fig. 3B).

5-HT upregulates CCL2 production in AM in a 5-HT\(_{2C}\) receptor-dependent manner. Cytokine and chemokine production in AM was screened with the dot-blot-based Mouse Cytokine Array. In AM stimulated with 5-HT (10\(^{-5}\) M) for 24 h, there was an increase in signal intensity over unstimulated controls mainly for CCL2 and to a lesser extent for TNF-\(\alpha\), CCL5 (RANTES), CXCL1 (KC), and CXCL2 (MIP-2) proteins (Fig. 4).

CCL2 protein levels in the cell culture supernatants were further quantified by ELISA. In line with the array data, 5-HT (at 10\(^{-5}\) but not 10\(^{-7}\) or 10\(^{-9}\) M; Fig. 5A) evoked a significant increase in CCL2 protein release, which was absent in AM isolated from 5-HT\(_{2C}\) receptor-deficient animals (Fig. 5B). Accordingly, qPCR revealed a significant 5-HT-induced increase in CCL2 mRNA level within 4 h in AM from wild-type littersmates, whereas no difference in CCL2 mRNA expression was observed in AM harvested from 5-HT\(_{2C}\) receptor-deficient mice (Fig. 5C and Table 1).
Baseline CCL2 release to supernatant of primary AEC and AM cultured for 24 h was much higher in AEC (1,648 ± 792.65 vs. 26.86 ± 5.14 pg/ml, respectively; data are expressed as means ± SD; P = 0.0003).

The 5-HT2C receptor drives 5-HT-induced transcriptional regulation in AM. To test the effect of 5-HT on the transcriptional regulation in AM, a whole mouse genome microarray analysis was performed. There were 53 genes regulated on 5-HT stimulation in wild-type AM (Fig. 6A). In contrast, no genes were differentially regulated in AM from 5-HT2C receptor-deficient animals, demonstrating the essential role of the 5-HT2C receptor in conferring 5-HT-induced transcriptional regulation in AM (Fig. 6B).

Table 1. The ΔΔCt with 95% confidence interval (CI) and fold change of the expression of selected genes on 5-HT treatment in AM isolated from wild-type (WT) and 5-HT2C receptor knockout animals (KO)

<table>
<thead>
<tr>
<th>Target Genes</th>
<th>Genotype</th>
<th>ΔΔCt ± SE</th>
<th>95% CI</th>
<th>Fold Change</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>ccl2</td>
<td>WT</td>
<td>0.67 ± 0.2</td>
<td>1.19 ... 0.15</td>
<td>1.59</td>
<td>0.0223</td>
</tr>
<tr>
<td>ccl2</td>
<td>KO</td>
<td>−0.28 ± 0.47</td>
<td>0.87 ... −1.42</td>
<td>0.82</td>
<td>0.575</td>
</tr>
<tr>
<td>spp1</td>
<td>WT</td>
<td>0.74 ± 0.24</td>
<td>1.4 ... 0.08</td>
<td>1.67</td>
<td>0.0357</td>
</tr>
<tr>
<td>spp1</td>
<td>KO</td>
<td>0.25 ± 0.24</td>
<td>0.9 ... −0.41</td>
<td>1.19</td>
<td>0.3585</td>
</tr>
<tr>
<td>nt5e</td>
<td>WT</td>
<td>1.18 ± 0.31</td>
<td>2.1 ... 0.25</td>
<td>2.27</td>
<td>0.0256</td>
</tr>
<tr>
<td>nt5e</td>
<td>KO</td>
<td>0.22 ± 0.32</td>
<td>1.05 ... −0.6</td>
<td>1.16</td>
<td>0.5131</td>
</tr>
<tr>
<td>myo1d</td>
<td>WT</td>
<td>1.83 ± 0.47</td>
<td>3.01 ... 0.66</td>
<td>3.56</td>
<td>0.0094</td>
</tr>
<tr>
<td>myo1d</td>
<td>KO</td>
<td>−1.02 ± 0.63</td>
<td>0.74 ... −2.78</td>
<td>0.49</td>
<td>0.1829</td>
</tr>
</tbody>
</table>

P values were calculated using Welch t-test. Ct, cycle threshold; 5-HT, serotonin; AM, alveolar macrophages.

Out of 53 genes regulated in the microarray analysis, 39 were upregulated, and 14 were downregulated. Of the upregulated genes, many were involved in cytokine activity (spp1), signal transduction involving G protein-coupled receptors (sphk1), biosynthetic processes (nt5e), or cell differentiation (csf1). Downregulated genes like ptges included such being involved in anti-inflammatory response, extracellular matrix organization (tgfb1), or peptidase activity (htra1). Genes marked on the volcano plot (Fig. 6A) were examined by qPCR. Their expression pattern on

Fig. 6. Genes regulated in AM on 5-HT stimulation. Microarray analysis of changes in gene expression of AM that were isolated from the wild-type (5-HT2C-WT; A) and 5-HT2C receptor-deficient (5-HT2C-KO; B) animals and treated with 5-HT (10^{-5} M) for 4 h. Volcano plots depict significance (p = P value) and fold change of gene expression on the y- and x-axis, respectively. Arbitrarily chosen differentially regulated genes (marked in A) were analyzed by qPCR, and hmbs served as reference gene (C). Data are presented as dot plots with indicated median values. *P < 0.05. SPP1, osteopontin; NT5E, ecto-5'-nucleotidase; MYO1D, myosin 1D.
Table 2. Correlation between coefficient values from microarray experiment and ΔCt values from quantitative PCR of selected genes

<table>
<thead>
<tr>
<th>Target Genes</th>
<th>Array Coefficient</th>
<th>PCR, ΔCt</th>
</tr>
</thead>
<tbody>
<tr>
<td>spp1</td>
<td>1.804</td>
<td>0.436598</td>
</tr>
<tr>
<td>ptpes</td>
<td>-0.574</td>
<td>-0.52207</td>
</tr>
<tr>
<td>m5se</td>
<td>1.129</td>
<td>0.669132</td>
</tr>
<tr>
<td>ccl2</td>
<td>0.3638</td>
<td>1.004898</td>
</tr>
<tr>
<td>csf1</td>
<td>1.486</td>
<td>0.568936</td>
</tr>
<tr>
<td>sphkl1</td>
<td>1.763</td>
<td>1.470716</td>
</tr>
<tr>
<td>il20rb</td>
<td>0.9992</td>
<td>0.202443</td>
</tr>
<tr>
<td>myo1d</td>
<td>0.9016</td>
<td>1.647529</td>
</tr>
<tr>
<td>flrt3</td>
<td>0.8337</td>
<td>0.033344</td>
</tr>
<tr>
<td>tgb1</td>
<td>-0.7531</td>
<td>-0.28022</td>
</tr>
<tr>
<td>icol</td>
<td>-0.5531</td>
<td>0.41871</td>
</tr>
<tr>
<td>slc7a2</td>
<td>-0.5071</td>
<td>-0.46499</td>
</tr>
<tr>
<td>htra1</td>
<td>-0.4427</td>
<td>-0.8837</td>
</tr>
</tbody>
</table>

AM were stimulated with $10^{-5}$ M 5-HT for 4 h. Positive values indicate upregulation and negative values downregulation of the respective genes in AM on 5-HT treatment. Correlation was measured with Pearson product-moment correlation coefficient as 1-sided test ($P = 0.0064, r = 0.667$).

average correlated with coefficient values obtained from microarray study (Table 2). Among genes examined by qPCR, spp1, m5se, and myo1d mRNAs were significantly increased on stimulation with 5-HT (Fig. 6C and Table 1).

DISCUSSION

In this study, we provide evidence for functional expression of 5-HT2C receptors on mouse AM, where 5-HT rapidly stimulates a rise in $[Ca^{2+}]_i$ and leads to changes in transcriptional profile and to the increased production of the monocyte chemoattractant CCL2.

The first indication of the 5-HT2C receptor expression on AM derived from our recent microarray study (57). Previous studies indicated a role of the 5-HT2 receptor subtypes 5-HT2A and 5-HT2B on murine pneumocytes in the development of bleomycin-induced pulmonary fibrosis (13) and of the 5-HT2A receptor in monocrotaline-induced pulmonary hypertension in rats (19). Studies focusing on human monocytes (6, 10), monocyte-derived dendritic cells (21), rat AM cell line NR8383, and human AM (35) explored the modulatory role of serotonergic AM stimulation. Indeed, a dot-blot-based assay and CCL2 ELISA revealed increased release of predominantly CCL2 but also CCL5, TNF-α, CXCL1, and CXCL2 into the medium after 24-h stimulation with 5-HT. Importantly, no increase in CCL2 production was observed in AM isolated from 5-HT2C receptor knockout animals, proving the involvement of the 5-HT2C receptor in those responses. In addition, we show that CCL2 mRNA is increased on 5-HT stimulation, and again this effect is 5-HT2C receptor-dependent.

CCL2 is a chemokine that drives monocyte recruitment to the lung under inflammatory conditions (34). This phenomenon has been confirmed in bacterial (55) and viral (18) models of lung inflammation where AEC mainly contribute to pathophysiological CCL2 levels. Interestingly, AM carry CCR2 receptors (33), indicating potential binding of CCL2, and are also known to produce CCL2, albeit in lower quantities than AEC. Depletion of resident AM, however, decreased alveolar neutrophil and monocyte recruitment in a combined LPS/CCL2 challenge (33).

Serotonin levels are elevated in inflamed tissues (36), and they reach 50–60 nmol/g, corresponding to ~5 × 10^{-5} M in mouse lung homogenates within minutes after LPS injection or antigen challenge in sensitized animals (41, 56). However, these data do not discriminate between intracellular and free 5-HT. Electron microscopy has indeed demonstrated platelet accumulation and degranulation under these conditions (56). In allergen-induced animal model of airway inflammation, extracellular 5-HT levels in BAL fluid are elevated to 1.7 × 10^{-6} M (27), which is close to the concentration we used in our experiments. Hence, significant 5-HT release from platelets is likely to occur under local inflammatory conditions in the lung where it may target AEC and AM. Contrary to AEC, AM...
express 5-HT$_{2C}$ receptors, the activation of which leads to increased CCL2 release. Interestingly, AEC express functional CCR2 receptors (5). Reaction of AEC to CCL2 might be important for chemotaxis and healing of wounds in the process of reepithelialization after lung injury (5) and may play a role in maintaining the lung microenvironment, e.g., by promoting cross talk between AM and AEC.

In view of the clear effect of 5-HT on expression of inflammatory mediators, we expanded analysis of transcriptional changes evoked by 5-HT in AM by whole mouse genome microarray analysis. Distinct differential gene expression induced by 5-HT, as observed in AM from wild-type mice, was abrogated in cells isolated from 5-HT$_{2C}$ receptor-deficient animals, demonstrating that 5-HT-induced transcriptional regulation in AM is exclusively conferred by the 5-HT$_{2C}$ receptor.

The differentially regulated genes identified by microarray analysis cover a broad spectrum of functions relevant to AM biology. Osteopontin (sppl), one of the genes upregulated in AM on 5-HT stimulation, is increased in interstitial lung diseases and promotes the accumulation of macrophages (42). In a murine model of allergen-induced airway remodeling, increased osteopontin production was associated with lung fibroblast activation and airway collagen deposition (22, 23). If transcriptional changes in osteopontin mRNA in AM translates to increased protein expression, it can be anticipated that 5-HT may play a role in local lung remodeling by acting on AM through 5-HT$_{2C}$ receptors, as it was suggested by Fabre et al. (13) for 5-HT$_{2AB}$ receptors on AEC. At the same time, however, ntSe, another upregulated AM gene in our study, is tissue-protective in the lung. Its protein product, ecto-5'-nucleotidase (CD73), converts AMP to adenosine and thereby is involved in the protection against lung injury induced by mechanical ventilation (11), LPS (43), and bleomycin (53). Concomitantly, osteopontin and other profibrotic mediators are increased in ntSe-deficient animals (53). Further studies have to be conducted to evaluate the role of the 5-HT-5-HT$_{2C}$ receptor signaling pathway in AM in lung pathology.

In conclusion, we identified 5-HT as a novel modulator of AM function, initiating [Ca$^{2+}$]$_i$ elevation, cytokine secretion, and transcriptional regulation. These effects are exclusively driven by the 5-HT$_{2C}$ receptor and provide the potential for selective intervention.

ACKNOWLEDGMENTS

We thank Dr. Pete Clifton (Department of Psychology, University of Sussex, Falmer, Brighton, United Kingdom) for providing us with knockout animals, Dr. Rory Motty for stimulating discussion, Martin Bodenbenner, Dagmar Hensel, and Petra Janssen for excellent technical assistance, and Karola Michael for help with the artwork.

GRANTS

This study was supported by the Deutsche Forschungsgemeinschaft (DFG; Excellence Cluster Cardio-Pulmonary System LO2711/4-1 Grant and IntKG 1062) and grants from the University Medical Center Giessen and Marburg, the National Network on Community-Acquired Pneumonia (CAPNETZ), and the Clinical Research Group Infectious Diseases (Grant 01 KI 0770).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES

ROLE OF SEROTONIN IN ALVEOULAR MACROPHAGE BIOLOGY


