Neuroendocrine Signaling Via the Serotonin Transporter Regulates Clearance of Apoptotic Cells*

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Serotonin (5-hydroxytryptamine; 5-HT) is a CNS neurotransmitter increasingly recognized to exert immunomodulatory effects outside the CNS that contribute to the pathogenesis of autoimmune and chronic inflammatory diseases. 5-HT signals to activate the RhoA/Rho kinase (ROCK) pathway, a pathway known for its ability to regulate phagocytosis. The clearance of apoptotic cells (i.e. efferocytosis) is a key modulator of the immune response that is inhibited by the RhoA/ROCK pathway. Because efferocytosis is defective in many of the same illnesses where 5-HT has been implicated in disease pathogenesis, we hypothesized that 5-HT would suppress efferocytosis via activation of the RhoA/ROCK. The effect of 5-HT on efferocytosis was examined in murine peritoneal and human alveolar macrophages, and its mechanisms were investigated using pharmacologic blockade and genetic deletion. 5-HT impaired efferocytosis in murine peritoneal and human alveolar macrophages. 5-HT increased phosphorylation of myosin phosphatase subunit 1 (Mypt-1), a known ROCK target, and inhibition of RhoA/ROCK reversed the suppressive effect of 5-HT on efferocytosis. Peroxineal macrophages expressed the 5-HT transporter and 5-HT receptors (R) 2a, 2b, but not 2c. Inhibition of 5-HT2a and 5-HT2b had no effect on efferocytosis, but blockade of the 5-HT transporter prevented 5-HT-impaired efferocytosis. Genetic deletion of the 5-HT transporter inhibited 5-HT uptake into peritoneal macrophages, prevented 5-HT-induced phosphorylation of Mypt-1, reversed the inhibitory effect of 5-HT on efferocytosis, and decreased cellular peritoneal inflammation. These results suggest a novel mechanism by which 5-HT might disrupt efferocytosis and contribute to the pathogenesis of autoimmune and chronic inflammatory diseases.

Background: Serotonin (5-HT) contributes to the pathogenesis of chronic inflammatory diseases known to have defects in apoptotic cell removal (i.e. efferocytosis).

Results: 5-HT impairs macrophage efferocytosis via 5-HT transporter-dependent activation of the RhoA/Rho kinase pathway.

Conclusion: Results show a novel mechanism by which 5-HT might disrupt resolution of inflammation.

Significance: Targeting the 5-HT transporter may have a therapeutic role in chronic inflammatory disorders.

Serotonin (5-hydroxytryptamine; 5-HT)² is a CNS neurotransmitter best known for its role regulating mood, appetite, sleep, and cognitive functions such as memory and learning (1). 5-HT also has a variety of important endocrine-like functions outside of the CNS, which include its ability to regulate vasomotor reaction, proliferation, osteoclastogenesis, insulin secretion, and platelet function (1–7). The literature also suggests that 5-HT is an important regulator of the immune system with a role in the pathogenesis of autoimmunity (8, 9), inflammatory bowel disease (10–12), pulmonary hypertension (13, 14), acute lung injury (15, 16), asthma (17), and chronic obstructive pulmonary disease (18–21).

5-HT is produced by two isoforms of tryptophan hydroxylase (tph), tph-2 in the CNS and tph-1 in the periphery(2). Despite its critical role in the CNS, over 95% of the 5-HT in the body is produced by enterochromaffin cells in the gut, where it regulates intestinal motility (6, 7). To a large extent, enterochromaffin cells also supply 5-HT to the rest of the body by secreting it into the blood where it is incorporated into platelets (via the 5-HT transporter) and delivered to distant organs and cells. Both structural and immune cells, such as macrophages, respond to 5-HT through one of 14 G protein-coupled receptors or via a 5-HT transporter-dependent process called serotonin (1, 2, 22, 23). Both mechanisms result in activation of the RhoA/Rho kinase (ROCK) pathway, which is a well known regulator of the cytoskeleton and phagocytosis, including phagocytosis of apoptotic cells (efferocytosis).

Apoptotic cells are recognized, eaten, and digested through efferocytosis, a distinctive process that maintains physiologic homeostasis and resolves inflammation following an infectious or non-infectious provocation (24–26). Efferocytosis utilizes a unique but redundant repertoire of bridging proteins and receptors that recognize externalized ligands on the surface of apoptotic cells. Interactions between efferocytosis receptors and apoptotic cell ligands have at least three significant effects: 1) phagocytosis of dying cells through activation of Rac-1; 2)
induction of mediators that suppress the innate immune response; and 3) suppression of adaptive immunity. Failed or dysfunctional efferocytosis has been implicated in the pathogenesis of chronic inflammatory diseases including, systemic lupus erythematous, rheumatoid arthritis, obesity, cardiovascular disease, neurodegenerative disease, and lung diseases such as cystic fibrosis, asthma, chronic granulomatous disease, and chronic obstructive pulmonary disease (24–31). In each disease, the cause(s) for impaired efferocytosis is complex, but in several cases the final common pathway involves activation of the RhoA/ROCK pathway (32–35), a well described negative regulator of efferocytosis (36). Because of the growing body of evidence linking 5-HT signaling with chronic inflammation and activation of the RhoA/ROCK pathway, this study was designed to determine the impact of 5-HT on efferocytosis and to define the mechanisms involved.

**MATERIALS AND METHODS**

*Reagents and Antibodies—*X-vivo10 media was obtained from Lonza (Walkersville, MD). Reagents purchased from Mediatech Inc. (Manassas, VA) included DMEM, RPMI 1640, PBS, and Hanks’ balanced salt solution. FBS was purchased from ATLANA Biologicals (Lawrenceville, GA). Serotonin (5-HT) and Y-27632 were obtained from Sigma and C3 transferase purchased from Tocris Biosciences (Bristol, UK). Fluoxetine HCl was from ICN Pharmaceuticals, Inc., Aurora, OH) and incubated for 1 h at room temperature before the experiments.

*Human Subjects—*The study was approved by and performed under institutional animal care and use committee-approved protocols at the animal facility of the National Jewish Health and University of Colorado Anschutz Medical Campus and National Jewish Health. Experiments were performed on 8–12-week-old 5-HTT-deficient (KO) mice (B6.129(Cg)-Slc6a4tm1Kpl/J) and age/sex-matched, C57BL/6J mice from Jackson Laboratories and National Jewish Health. Written informed consent was obtained from each subject.

*Experimental Animals—*Mice were housed and studied under institutional animal care and use committee-approved protocols at the animal facility of the National Jewish Health and University of Colorado Anschutz Medical Campus and National Jewish Health. Experiments were performed on 8–12-week-old 5-HTT-deficient (KO) mice (B6.129(Cg)-Slc6a4tm1Kpl/J) and age/sex-matched, C57BL/6J mice from Jackson Laboratories (Bar Harbor, ME) (37–39).

*Western Blotting—*Immunoblot analysis was carried out as described previously with some modifications (32). Briefly, macrophages (1.0 × 10⁶ cells/well) were plated in each well of a 6-well tissue culture plate. Following stimulation, the cells were lysed in RIPA buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM dithiothreitol, 0.5% Triton X-100, and 1× protease inhibitor mixture set I), resolved on 7.5% SDS-PAGE, and blotted onto nitrocellulose membranes. The membranes were probed with primary antibodies at 4 °C overnight and incubated with
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horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Proteins were visualized by enhanced chemiluminescence (Thermo Scientific, Rockford, IL) according to the manufacturer’s instructions. Equal loading of proteins in each lane was confirmed by reprobing with the corresponding antibodies against the native proteins or β-actin. The results shown are representative of at least three separate experiments.

Neurotransmitter Transporter Uptake Assay—Neurotransmitter uptake was assessed using the Neurotransmitter Transporter Uptake Assay Kit (Molecular Devices, Sunnyvale, CA). Experiments were performed according to the manual. Briefly, TG-elicited peritoneal macrophages from 5-HTT KO mice and wild-type mice were cultured in a 96-well plate at 1 × 10^5 cells per well in Hanks’ balanced salt solution. The neurotransmitter tracer was added to each well and the plate was incubated for 60 min in 5% CO_2 at 37 °C. The tracer becomes fluorescent when internalized through the 5-HT, dopamine, and norepinephrine transporters. The fluorescent signal was read using a Synergy H1 Hybrid Reader (Biotek, Winooski, VT). Specificity for uptake through the 5-HT transporter was determined using genetic deletion of the 5-HT transporter.

RNA Isolation and Real-time PCR—RNA was extracted using RNeasy Mini Kit (Qiagen, Valencia, CA). The protocol utilized the on-column DNase digestion with RNase-free DNase set (Qiagen). The on-column DNase digestion was also performed per the manufacturer’s instructions. The RNA quantity and quality was determined using the NanoDrop Spectrophotometer (Thermo Scientific). 0.5 μg of total RNA was reverse transcribed utilizing the High Capacity RNA to cDNA kit (Applied Biosystems, Foster City, CA). For quantitative real-time PCR, 3 μl of cDNA was brought up to 9 μl with nuclease-free water and combined with 1 μl of TaqMan Gene Expression Assay and 10 μl of TaqMan Gene Expression Master Mix (Applied Biosystems). Assays were performed in triplicate under standard real-time PCR conditions (95 °C for 10 min and 40 cycles of 95 °C for 15 s followed by 60 °C for 1 min) using a 7300 Real-time PCR system (Applied Biosystems) with sequence detection software. Inventoried TaqMan Gene Expression Assays (Applied Biosystems) were used to measure mRNA expression levels for glyceraldehyde-3-phosphate dehydrogenase (GAPDH, assay ID Mm03302249_g1), HTR2a (htr2a, assay ID Mm00555764_m1), HTR2b (htr2b, assay ID Mm00434123_m1), and 5-HT transporter (SLC6A4, assay ID Mm00439391_m1). GAPDH was used as the endogenous control.

Statistical Analysis—Data are presented as mean ± S.E., in bar graphs for repeated observations from two or more separate experiments, or in scatter plots for independent observations from two groups (5-HTT KO and wild-type controls). For neurotransmitter uptake, paring was indicated in scatter plots for paired observations from 5-HTT KO and wild-type controls. The means for repeated observations were conservatively compared using ANOVA. When ANOVA indicated significance, the Dunnett’s test was used to compare groups versus a reference group adjusting for multiple comparisons. For two-way ANOVA, interaction, genotype, and treatment effects were tested and Tukey-Kramer post hoc analysis was conducted. Significant tests of percent differences from media control were conducted for F-Tests and adjusting for multiple testing using Bonferroni t-statistics. (Data are presented as mean ± S.E. in percent of control where media control at 100% is shown as a reference.) The means for independent observations between 5-HTT KO and wild-type controls were robustly compared using the Mann-Whitney test. Paired t test was used for the paired data for neurotransmitter uptake. All data were analyzed using Prism statistical software for the Macintosh (GraphPad Software, Inc., La Jolla, CA) and SAS vs9.2 (SAS Institute, Cary, NC).

RESULTS

Effect of 5-HT on Efferocytosis—To investigate the effect of 5-HT on efferocytosis, murine resident and TG-elicited peritoneal macrophages were isolated and co-cultured with apoptotic murine thymocytes or carboxylated beads in the presence or absence of 5-HT. Carboxylated beads are frequently used as an apoptotic cell surrogate because they are negatively charged and are ingested via an efferocytosis-like mechanism (36). Carboxylated beads are also indigestible, so they can distinguish between effects on ingestion versus digestion (41). TG-elicited peritoneal macrophages were isolated and cultured for 24 h in serum-free Xvivo10 containing 0.1% low endotoxin BSA and various concentrations of 5-HT (i.e. 0–1,000 nM). Peritoneal macrophages were then co-cultured with apoptotic thymocytes at a 10:1 ratio (apoptotic cell to macrophage) for 60 min or carboxylated beads at a 2:1 ratio (beads to macrophages) for 15 min, washed, stained, and assessed for ingestions by blinded, visual assessment. Examples of ingestion and binding of apoptotic thymocytes by peritoneal macrophages are shown in Fig. 1, A and B. 5-HT inhibited ingestion of both apoptotic cells and carboxylated beads by both resident and TG-elicited peritoneal macrophages (Fig. 1).

Several groups have studied the effect of 5-HT on phagocytosis of targets that are unrelated to efferocytosis by murine peritoneal macrophages, such as bone marrow-derived macrophages and microglia, but the effects have been variable (42–44). We performed similar experiments using resident and TG-elicited peritoneal macrophages and found that 5-HT inhibited ingestion of latex beads, but had no effect on ingestion of IgG-opsonized erythrocytes through the Fcγ-receptor (Fig. 2). These results indicate that the suppressive effect of 5-HT on efferocytosis is not uniform across all mechanisms of phagocytosis.

5-HT Inhibits Efferocytosis through Activation of the RhoA/ROCK Pathway—The RhoA/ROCK pathway is a potent negative regulator of efferocytosis. 5-HT activates the RhoA/ROCK pathway, leading us to hypothesize that 5-HT suppresses efferocytosis via a RhoA/ROCK-dependent mechanism. To test this hypothesis, TG-elicited peritoneal macrophages were treated with 5-HT in the presence or absence of the specific RhoA inhibitor, C3 transferase, or the specific ROCK inhibitor, Y-27632. C3 transferase and Y-27632 both reversed the ability of 5-HT to inhibit efferocytosis (Fig. 3, A and B). ROCK is a serine/threonine kinase that phosphorylates myosin phosphatase-1 (Mypt-1) at Thr-696 (25). Our results demonstrated that 5-HT increases phosphorylation of Mypt-1 at Thr-696 (Fig. 3C).
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Effect of 5-HT Receptor and 5-HTT Blockade on 5-HT-impaired Efferocytosis—To determine the mechanism for 5-HT impaired efferocytosis, apoptotic cell ingestion by peritoneal macrophages was examined following 5-HT receptor blockade using the 5-HTR2a antagonist, R-96544, the 5-HTR2b antagonist, RS-127445, and the 5-HTT antagonist, fluoxetine. 5-HT impaired efferocytosis, but was unaffected by either R-96544 or RS-127445 (Fig. 5, A and B). In contrast, fluoxetine prevented the ability of 5-HT to suppress efferocytosis by TG-elicited peritoneal macrophages (Fig. 5C). These data suggest that 5-HT impairs efferocytosis via a 5-HTT-dependent mechanism, and not through a 5-HTR2a or -2b-dependent mechanism.

Effect of 5-HTT Deficiency on 5-HT-impaired Efferocytosis by Murine Peritoneal Macrophages—The ability of fluoxetine to prevent 5-HT from suppressing efferocytosis suggests involvement of the 5-HTT. However, inhibitor experiments can never be definitive due to recognized and unrecognized off-target effects. To address this critical issue, experiments were performed using peritoneal macrophages from 5-HTT KO and control mice. Rudd and colleagues (45) previously used real-time PCR, Western blot analysis, and [3H]5-HT uptake to demonstrate the presence of a functional 5-HT transporter in peritoneal macrophages. We confirmed the presence of a functional 5-HT transporter in peritoneal macrophages using a neuroendocrine uptake assay, where a masked tracer becomes

in a Y-27632-sensitive manner (Fig. 3D), supporting that 5-HT increases ROCK activity.

Expression of 5-HT Receptors and Transporter in Murine Peritoneal Macrophages—5-HT has been shown to activate Rho GTPases by interacting with one of 14 known G protein-coupled receptors (23) or via a 5-HT transporter-dependent mechanism (5). We queried the open public gene array database of the Immunologic Gene Project to determine which of the 5-HT receptors or the 5-HTT are expressed in mouse peritoneal macrophages. The Immgen database showed that the major 5-HT receptors expressed on resident or TG-elicited murine peritoneal macrophages were 5-HT receptor 2a (5-HTR2a), 5-HT 2b (5-HTR2b), and the 5-HTT. We confirmed the expression of 5-HTR2a, 5-HTR2b, and 5-HTT by real-time PCR (Fig. 4A) and Western blotting (Fig. 4, B–D). In contrast, 5-HTR2c was not expressed (Fig. 4A).
fluorescent upon internalization through the 5-HT, dopamine, or the norepinephrine transporters. Specificity of the assay depends on the use of specific inhibitors or preferably by genetic deletion of a given transporter. Results showed that internalization of the tracer by 5-HTT KO peritoneal macrophages was decreased compared with wild-type controls (Fig. 6A). To confirm the involvement of 5-HTT in the regulation of efferocytosis, TG-elicited peritoneal macrophages from wild-type and 5-HTT KO mice were treated with 5-HT for 24 h and then assessed for their ability to ingest apoptotic thymocytes. As was seen with fluoxetine (Fig. 5C), 5-HT inhibited efferocytosis by wild-type macrophages, but did not suppress efferocytosis by macrophages from 5-HTT KO mice (Fig. 6B). Similarly, 5-HT increased phosphorylation of Mypt-1 at Thr-696 in macrophages from wild-type mice, but not from 5-HTT KO mice (Fig. 6C). Together these results indicate that 5-HT acts through its transporter to activate the Rho kinase pathway and inhibit efferocytosis in murine peritoneal macrophages.

**Effect of 5-HTT Deficiency on Cellular Peritoneal Inflammation—**Efferocytosis promotes the resolution of inflammation, leading to the hypothesis that 5-HTT deficiency may accelerate the resolution phase of the inflammatory response by preserving efferocytosis. To examine this hypothesis, cell counts were performed on peritoneal exudates 4 days post-intraperitoneal injection with TG in wild-type and 5-HTT KO mice. These experiments showed a decrease in neutrophils (Fig. 6D) in 5-HTT KO mice versus wild-type controls. In contrast, 5-HTT deficiency had no effect on macrophages (wild-type: 2.1 \times 10^{11} \pm 3.9 \times 10^{10} versus 5-HTT KO: 1.5 \times 10^{11} \pm 1.8 \times 10^{10}, p = 0.23, Mann Whitney test). These results suggest that sup-
pression of 5-HTT activity promotes the resolution phase of inflammation.

5-HT Inhibits Efferocytosis by Human Alveolar Macrophages through a 5-HTT and ROCK-dependent Process—Because the repertoire of expressed 5-HT receptors and transporter may vary significantly between species and macrophage type, we sought to determine whether 5-HT has similar effects on efferocytosis by human alveolar macrophages. Like TG-elicited peritoneal macrophages, normal human alveolar macrophages expressed 5-HTT protein by Western blot (Fig. 7A). In addition, 5-HT suppressed efferocytosis of apoptotic Jurkat T-cells, an effect that was reversed by the 5-HTT inhibitor, fluoxetine (Fig. 7B), and the ROCK inhibitor, Y-27632 (Fig. 7C). Together, these results suggest that 5-HT inhibits efferocytosis by both human and murine macrophages via a 5-HTT/ROCK-dependent mechanism.

DISCUSSION

Our study demonstrates for the first time the potential of the neuroendocrine system to regulate efferocytosis and thereby influence the maintenance of homeostasis as well as the resolution of inflammation. Our data confirm the hypothesis that 5-HT inhibits efferocytosis by activating the RhoA/ROCK pathway, a well known, potent, negative regulator of efferocytosis (32–36). Unexpectedly, 5-HT did not inhibit efferocytosis via one of its many G protein-linked receptors, but rather suppressed efferocytosis through a 5-HT transporter-dependent mechanism, a protein that is better known as a regulator of neurotransmission than of macrophage biology. Because over 95% of 5-HT is produced outside of the CNS (2, 7), these results may have wide-ranging implications for health and disease.

Results show that 5-HT effectively inhibits phagocytosis of apoptotic cells, carboxylated beads, and latex beads by peritoneal macrophages, but has no effect on ingestion of IgG-opsonized erythrocytes via the Fc/H9253 receptor. Few prior studies have investigated the ability of 5-HT to regulate phagocytosis and none have examined efferocytosis or ingestion through the Fc/H9253 receptor. Existing studies are conflicting. For example, Sternberg et al. (44) explored the ability of 5-HT to regulate...
phagocytosis of latex beads by bone marrow-derived macrophages isolated from SJL mice. They found that 5-HT enhanced latex bead ingestion when cells were simultaneously exposed to small concentrations of IFNγ but inhibited ingestion when exposed to larger concentrations. In contrast, Freire-Garabal et al. (42) found that a short exposure to 5-HT increased latex bead phagocytosis by resident peritoneal macrophages from BALB/c mice. More recently Krabbe et al. (43) demonstrated that 5-HT inhibits ingestion of microspheres by microglia, resident macrophages of the CNS. These studies differ substantially from ours in regard to mouse strain, macrophage type, and 5-HT exposure (e.g. time and concentration). Together, these differences make a direct comparison difficult. More recently, an intriguing study demonstrated the importance of 5-HT in effective erythrophagocytosis, where the absence of 5-HT decreased differentiation of erythroid precursors and increased phagocytosis of erythrocytes (46). Like apoptotic cells, aging erythrocytes externalize phosphatidylserine on their outer membrane allowing them to be recognized and removed by phagocytes (47–50). These findings suggest that physiologic
levels of 5-HT regulate erythropoiesis, in part by inhibiting their recognition and removal from circulation by phagocytes. 5-HT may exert its effects through any of the 14 known G protein-linked 5-HT receptors or through the 5-HTT (7, 22), suggesting a sizable dimension of potential effects. Specific macrophage populations contain their own unique repertoire of 5-HT receptors. For example, our data demonstrate that peritoneal macrophages express 5-HTR2a and 5-HTR2b, but do not express 5-HTR2c. In contrast, murine alveolar macrophages express much higher levels of 5-HTR2c and lower levels of 5-HTR2a and 5-HTR2b (51). Murine alveolar macrophages also express higher amounts of 5-HTT than do peritoneal macrophages (www.immgen.org). Despite the presence of 5-HTR2a and 5-HT2b, 5-HT appears to inhibit efferocytosis through a 5-HTT-dependent mechanism.

The results support the conclusion that 5-HT inhibits efferocytosis via a RhoA/ROCK-dependent mechanism. 5-HT is known to activate the RhoA/ROCK pathway through any of its G protein-linked receptors or through a recently described 5-HTT-dependent mechanism, called serotonylation (5, 23, 52). Our data indicate that 5-HT impairs efferocytosis via a 5-HTT-dependent mechanism, not by activating 5-HTR2a or 5-HTR2b, suggesting that serotonylation may be involved. During serotonylation, transglutaminase-2 transamidates proteins by covalently linking 5-HT to glutamine residues.

There are multiple potential mechanisms by which serotonylation may regulate RhoA activity. First, like other Rho GTPases, RhoA may be directly serotonylated (5, 52). Serotonylated GTPases are resistant to GTP hydrolysis, rendering them constitutively active. Serotonylated RhoA has been demonstrated to be a critical mechanism for normal hemostasis by regulating platelet attachment (5). Increased serotonylated RhoA has also been implicated in the pathogenesis of pulmonary hypertension via enhanced activation of ROCK and increased vasoconstriction (14). Second, 5-HT receptor-associated G proteins are suggested to be targets for activation through serotonylation (2). This would indicate that 5-HT receptors may activate RhoA through mechanisms that are separate from traditional receptor/ligand interactions. Finally, two recent studies demonstrated that the extracellular matrix glycoprotein, fibronectin, is a target for serotonylation (53, 54). Although no direct link for RhoA/ROCK activation has been made, fibronectin is known to bind both α5β1 and α5β3 integrins, which results in RhoA activation (55).

The studies presented here represent the beginning of our understanding of the mechanisms by which 5-HT regulates efferocytosis. Further studies will be necessary to determine the involvement of serotonylation and identify whether the target of serotonylation is RhoA or one of the other possible targets that regulate RhoA activity. Likewise, the ability of 5-HT to impair efferocytosis will need to be studied in the context of human diseases with impaired efferocytosis (and their animal models) as outlined above, in particular those diseases where 5-HT has already been implicated in disease pathogenesis. It is this connection that will suggest the therapeutic potential of pharmacologically regulating the 5-HT pathway.

In summary, we have shown the ability of neuroendocrine signaling via 5-HT to inhibit efferocytosis via a pathway that

![Diagram of Serotonin Regulates Phagocytosis of Apoptotic Cells](Image)

**FIGURE 8. Proposed model for the ability of 5-HT to suppress efferocytosis.** 5-HT impairs efferocytosis by activating RhoA and ROCK. 5-HT appears to exert its effect on efferocytosis through the 5-HTT transporter and not through 5-HTT receptor 2a or 5-HTT receptor 2b (red X).

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