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Preliminary report

The anti-inflammatory effects of the 5-HT_3 receptor antagonist tropisetron are mediated by the inhibition of p38 MAPK activation in primary human monocytes

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ABSTRACT

Background: There is evidence from human and animal research that 5-hydroxytryptamine (5-HT) 3 receptor antagonists, particularly tropisetron, exert analgesic and anti-inflammatory activity. We have demonstrated that tropisetron inhibited lipopolysaccharide (LPS)-stimulated tumor necrosis factor (TNF)alpha and interleukin-(IL-)1beta release in primary human monocytes. The underlying mechanisms of these effects have not been investigated in detail so far.
Methods: The molecular mechanisms of the anti-inflammatory effects of tropisetron were investigated in human primary monocytes *in vitro* by studying IL-1beta and TNFalpha mRNA levels by PCR and reporter gene assay and by elucidating the phosphorylation of p38 mitogen activated kinase (MAPK) by Western blot.
Results: The steady state levels of IL-1beta and TNFalpha mRNA in LPS-activated human peripheral monocytes and the transcriptional activity of the TNFalpha promoter were not inhibited by tropisetron, suggesting that the inhibitory activity of this 5-HT_3 receptor antagonist takes place at the post-transcriptional level. Additionally, we found that tropisetron prevents the phosphorylation and thus activation of the p38 MAPK, which is involved in post-transcriptional regulation of various cytokines.
Conclusion: Our data indicate that the anti-inflammatory effects of the 5-HT_3 receptor antagonist tropisetron, as shown *in vivo*, are possibly mediated by a selective inhibition of pro-inflammatory cytokines at the post-transcriptional level. 5-HT_3 receptor antagonists are therefore a new and promising therapeutic option. New and more selective – in respect to the 5-HT_3 subtypes – 5-HT_3R antagonists might be a future perspective in the pharmacological treatment of inflammatory diseases.

1. Introduction

5-Hydroxytryptamine-3 (5-HT_3) receptor antagonists currently have primary therapeutic indications for emesis in cancer chemotherapy and irritable bowel syndrome [1], and there is some evidence, albeit controversial, that they may be useful for treatment of a range of psychiatric disorders [2]. Some studies also provided evidence for an analgesic as well as an antiphlogistic effect of 5-Hydroxytryptamin-3 (5-HT_3) receptor antagonists, particularly for tropisetron: the pain relieving effect of these substances in patients was initially demonstrated in fibromyalgia [3–5], a syndrome characterized by generalized chronic pain of the musculoskeletal system and proven in a prospective, multicenter, double-blind, parallel-group, dose-finding study, with 418 fibromyalgia patients [6]. The clinical results achieved by local application of tropisetron in various inflammatory and non-inflammatory rheumatic diseases, such as arthritides of different origin, periarthropathies, tendinopathies and trigger points [6–9], indicate that 5-HT_3 receptor antagonists apparently have an antiphlogistic effect in addition to their analgesic action. This antiphlogistic action might also explain why the effect of local injections of tropisetron lasts much longer in tendinopathies, for example, than that of well-known local anesthetics [9,10]. It may also be the reason why local injections of tropisetron are just as effective as a combination of local anesthetics and corticosteroids in the therapy of all above-mentioned diseases, a finding that was corroborated by several clinical studies [8,9]. But also experimental studies suggest that 5-HT_3 receptor antagonists possess both an analgesic and an antiphlogistic effects. Experimentally induced acute and chronic

Abbreviations: 5-HT, 5-Hydroxytryptamine; IL, interleukin; TNF, tumor necrosis factor; PG, prostaglandin; MMP, matrix metalloprotease; PMA, phorbol myristate acetate; PHA, phytohemagglutinin; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase.

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inflammatory pain [11–14] as well as chemically induced pain [15,16] responded well to 5-HT_3 receptor antagonists, probably because the latter inhibit nociceptor stimulation.

1.1. 5-HT_3 receptors

5-HT_3 receptors occur mainly in the peripheral nervous system, particularly on nociceptive sensory neurons, and on autonomic and enteric neurons, on which serotonin (5-HT) exerts a strong excitatory effect [17]. The central 5-HT_3 receptors are concentrated in regions that are involved, among other things, in integration of the vomiting reflex and pain processing [18]. Unlike other 5-HT receptors, which are G-protein-coupled receptors, 5-HT_3 receptors are exceptional in being directly linked to membrane ion channels, and do not involve any second messenger step in their transduction mechanism. The 5-HT_3 receptor is a ligand-gated cation channel belonging to the nicotine/GABA receptor superfamily. The ion channel itself is an oligomeric complex composed of five subunits [19]. Two different 5-HT_3 receptor subunits, 5-HT_{3A}, and 5-HT_{3B} were identified in the nineties, and recently 3 other human 5-HT_3 receptor-like genes, *HT3C*, *HT3D*, and *HT3E*, have been cloned [20]. It has been shown that the 5 subunits are differentially expressed in different tissues, which might explain the complexity of observed pharmacological and physiological responses found with medications targeting 5-HT_3 receptors [20]. Activation of 5-HT_3 receptor is followed by rapid depolarization of the peripheral or central neurons, causing a rapid increase in free cytosolic Ca^{2+} levels by inducing Ca^{2+} influx and mobilization of intracellular Ca^{2+} stores, modulating the release of various neurotransmitters and neuropeptides such as dopamine, cholecystokinin, acetylcholine, GABA, substance P or 5-HT itself [16].

2. Objective

Clinical and experimental observations concerning the analgesic and antiphlogistic effects of 5-HT_3 receptor antagonists may be of high significance since they could offer new options for understanding inflammatory processes and perhaps for adding new therapeutic strategies in a variety of pathophysiological states. We were able to show that the 5-HT3A receptor is expressed in human monocytes and that 5-HT3 antagonists such as tropisetron inhibit LPS-induced IL-1beta and TNFalpha release, but the molecular mechanisms of this anti-inflammatory action were not elucidated [21,22].

Since the underlying mechanisms leading to these beneficial effects *in vitro* and *in vivo* are not understood, especially the molecular mechanisms by which 5-HT_3 receptor antagonists reveal its potential anti-inflammatory activity, we tried to identify the effects of 5-HT_3 receptor antagonist tropisetron on mRNA expression of IL-1beta and TNFalpha and on the activation of p38 MAPK in the well established primary human monocyte model used to test anti-inflammatory drugs.

3. Materials and methods

3.1. Cell lines and reagents

The lymphoid cell line Jurkat (ATCC, Rockville, MD, US) was maintained in exponential growth in RPMI 1640 (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) supplemented with 10% heat-inactivated FCS (Sigma-Aldrich Chemie), 2 mM L-glutamine, 1 mM HEPES (Sigma-Aldrich Chemie) and the antibiotics penicillin (100 U/ml) and streptomycin (100 μg/ml) (Invitrogen, Barcelona, Spain). Tropisetron (Navoban®, Novartis, Basel, Switzerland) was purchased from Sigma or from a local pharmacy (no differences were observed between the Sigma-Aldrich pure compound and the clinically used one). Lipopolysaccharide (LPS) from *Escherichia coli* and all other reagents were obtained from Sigma (Sigma-Aldrich Chemie GmbH).

3.2. Isolation of human peripheral monocytes

Monocytes from healthy human donors were prepared following a standardized protocol (Ficoll gradient preparation, GE Healthcare, Freiburg, Germany) using a completely endotoxin-free cultivation [23,24]. Using 50 ml tubes, 25 ml Ficoll was loaded with 25 ml blood (buffy coats) from healthy blood donors. The gradient was established by centrifugation at 1800 rpm, 20 °C for 40 min with slow acceleration and deceleration. Peripheral blood mononuclear cells in the interphase were carefully removed and resuspended in 50 ml pre-warmed phosphate buffered saline (Cell Concepts, Umkirch, Germany), followed by centrifugation for 10 min at 1600 rpm and 20 °C. The supernatant was discarded and the pellet was washed in 50 ml PBS and centrifuged as described above. The pellet was then resuspended in 50 ml RPMI-1640 low-endotoxin medium supplemented with 10% human serum (PAA Coelbe, Germany). After counting the number of cells in a particle counter (Euro Diagnostics, Krefeld, Germany), cells were seeded in 24-well plates for ELISA or in 6-well plates for RNA analysis (1–2 mio. cells/well) and incubated at 37 °C/5% CO_2. The medium and the non-adherent cells (lymphocytes) were removed and fresh RPMI-1640 medium containing 1% human serum was added. Monocytes were thus ready to be used for the experiments.

3.3. Transient transfections and Luciferase assays

Jurkat cells (10^6/ml) were transiently transfected with the TNF-Luc (−1185 pTNFα-Luc) plasmid, which was previously described (Rhoades et al., 1992). The transfections were performed using Lipofectin™ reagent (Invitrogen, Karlsruhe, Germany) for 24 h, according to the manufacturer's recommendations. After incubation with tropisetron for 30 min, transfected cells were stimulated for 6 h with PMA (20 ng/ml) plus PHA (1 μg/ml). Then the cells were lysed in 25 mM Tris-phosphate pH 7.8, 8 mM $MgCl_2$, 1 mM DTT, 1% Triton X-100, and 7% glycerol. Luciferase activity was measured using an Autolumat LB 9501 (Berthold Detection Systems GmbH, Pforzheim, Germany) following the instructions of the luciferase assay kit (Promega, Madison, WI, US). The background obtained with the lysis buffer was subtracted in each experimental value and the specific transactivation expressed as a fold induction over untreated cells.

3.4. RNA extraction and PCR analysis

Total RNA was extracted using the guanidine isothiocyanate method [25]. For RT-PCR, 1 μg total RNA was reverse transcribed using MuMLV-reverse transcriptase (Gibco, Eggenstein, Germany) and random hexamers. Primers were designed using PrimerSelect Software (DNA Star Inc., Madison, WI US). PCR was then carried out adding Taq polymerase (Promega, Madison, WI, US) and a pair of primers for human IL-1beta (sense: 5′-CAG GCC GCG TCA GTT GTT GT-3′, anti-sense: 5′-TTA TAT CCT GGC CGC CTT TGG TC-3′, 57.5 °C, 35 cycles) and human TNFalpha (sense: 5′-CGA ACC CCG AGT GAC AAG-3′, anti-sense: 5′-GAA GAC CCC TCC CAG ATA GAT-3′, 58.6 °C, 35 cycles). PCR products were then separated electrophoretically on a 2% agarose gel.

3.5. Western blot analysis

For stimulation experiments, cells were exposed to LPS (10 ng/ml) in the presence or absence of tropisetron for the indicated periods of time. Cells were then washed with ice-cold PBS (Cell Concepts, Umkirch, Germany). Cell lysis was conducted using SDS-sample buffer (42 mM Tris–HCl pH 6.8, 1.3% sodium dodecyl sulfate buffer, 100 μm orthovanadate, 6.5% glycerin), and samples were homogenized by repeated pipetting, followed by an incubation step at 95 °C for 5 min. Protein content was determined using the bicinchoninic acid method (BCA protein assay kit) (KFC Chemikalien, München, Germany). 35–55 μg cell protein was subjected to SDS-PAGE on a 12% gel under reducing conditions. Proteins were transferred onto a polyvinylidene