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Regulation of neutrophil phagocytosis of *Escherichia coli* by antipsychotic drugs

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ABSTRACT

Antipsychotic drugs (APDs) have been used to ease the symptoms of schizophrenia. APDs have recently been reported to regulate the immune response. Our previous studies revealed that the atypical APDs risperidone and clozapine and the typical APD haloperidol can inhibit the phagocytic ability of macrophages. Our research next determined the effects of APDs on the phagocytic ability of neutrophils, which are the most abundant type of white blood cells in mammals. Here we provide evidence that clozapine and haloperidol can induce increased phagocytic uptake of *Escherichia coli* by differentiated HL-60 cells and by purified human neutrophils. Furthermore, clozapine and haloperidol can increase the myeloperoxidase activity and IL-8 production in neutrophils. Our results also show that clozapine can inhibit *E. coli* survival within differentiated HL-60 cells. Furthermore, clozapine and haloperidol are shown to enhance cell surface Mac-1 expression and the activated AKT signaling pathway in purified neutrophils exposed to *E. coli*. These results indicate that clozapine and haloperidol can increase the phagocytic ability of neutrophils by increasing AKT activation when cells are exposed to bacteria.

1. Introduction

Antipsychotic drugs (APDs) are a group of psychiatric medications used to ease the symptoms of psychotic disorders. First-generation antipsychotics, known as typical antipsychotics, such as chlorpromazine and haloperidol, bind mainly to dopamine D2 receptors [1–3] and were discovered in the 1950s. Most second-generation drugs, known as atypical antipsychotics, such as risperidone and clozapine, bind not only to dopamine D2 receptors, but also to type II serotonin receptors (5-HTRs), dopamine D3 receptor, and dopamine D4 receptor [4–8] and were developed later.

Over the years, APDs have been shown to regulate the immune response, and this is involved in the side effects of the medications. Changes in the serum levels of interleukin (IL)-1β, IL-2, IL-6, IL-8, IL-10, and interferon (IFN)-γ as well as regulation of the expression of the receptors for IL-2 and IL-6 have been observed in patients receiving APDs [9–13]. APDs have also been shown to affect immune cell responses. Clozapine and risperidone can inhibit Th1 differentiation, which decreases IFN-γ production by T cells [14,15]. Risperidone has been shown to modulate chemokine/cytokine release by dendritic cells, which regulate Th1/Th2 differentiation [16]. Decreased phagocytic ability of macrophages has been reported for cells treated with risperidone, clozapine, or haloperidol [17].

Neutrophils are the most abundant white blood cells in the circulation and act as the first line of host defense against invasion by microbial pathogens [18,19]. Many receptors on neutrophils mediate neutrophil phagocytosis. For example, toll-like receptor 4 (TLR4), Fc-γ receptors (CD16), complement receptors CR1 (CD35), and Mac-1 (CD11b/CD18) play important roles in neutrophil-mediated phagocytic uptake of pathogens [20,21]. Previous studies have demonstrated that the intracellular PI3K/AKT and MAPK signaling pathways are involved in phagocytosis by neutrophils [22–26].

Drug-induced neutropenia (neutrophil count $<1.5 \times 10^9$/L) or agranulocytosis (neutrophil count $<5.0 \times 10^8$/L) has been observed in patients treated with APDs [27–35]. Previous studies have emphasized the possible mechanism of side effects observed in APD-treated patients. Formation of immature neutrophils has been reported in patients who are on APD medications [36,37]. Also, our recent study indicated that the treatment of dendritic cells with risperidone may induce their production of TNF-α, which subsequently promotes death of neutrophils [16]. Both of these phenomena may be causes of the neutropenia or agranulocytosis induced by APDs.

In addition to functions of T cells and macrophages, neutrophil functions may also be regulated by APDs. Due to the short lifespan of purified neutrophils [38,39], it is difficult to study the effect of APDs on purified neutrophils. Many studies use reagents, such as DMSO or all-trans retinoic

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acid (ATRA), to induce differentiation of HL-60 cells [40,41], which can be used as a neutrophil model [42–44]. Comparisons of differentiated HL-60 cells and purified neutrophils have also been investigated [42,45].

To study the effects of APDs on neutrophils, we first examined the effects of a typical APD (haloperidol) and of atypical APDs (risperidone and clozapine) on the phagocytic uptake of *Escherichia coli* (*E. coli*) by ATRA-treated differentiated HL-60 cells and purified neutrophils, which were obtained from participants who had no history of APD treatment. We also examined the effects of APDs on cell surface markers and on the activation of AKT/MAPK signaling cascades in purified neutrophils. We found that purified neutrophils treated in vitro with clozapine or haloperidol show enhanced phagocytic uptake of bacteria. Furthermore, increased Mac-1 expression and activation of AKT signaling were also observed in neutrophils treated with clozapine and haloperidol.

2. Materials and methods

2.1. Reagents

Clozapine and haloperidol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Risperidone was obtained from Janssen-Pharmaceutica (Beerse, Belgium).

2.2. Preparation of ATRA-treated HL-60 cells

HL-60 cells, a human promyelocytic leukemia cell line, were maintained in growth medium consisting of RPMI-1640 supplemented with 10 mM HEPES, 1.5 g/L $NaHCO_3$, 4.5 g/L glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 units/mL penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum (FBS), and also 1 μM ATRA for 5 days to induce HL-60 cell differentiation. On the second day of ATRA treatment, APD was added to the culture medium and was replenished daily during HL-60 cell differentiation.

2.3. RNA isolation and quantitative real-time reverse transcription PCR

The levels of gene expression in control HL-60 cells, ATRA-treated HL-60 cells, and purified human neutrophils were measured using real-time quantitative PCR (Q-PCR). Total RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA was then reverse-transcribed to cDNA by incubation at 37 °C for 1 h in 20 μL of a mixture containing 5 μg of total RNA, 1 U of Moloney murine leukemia virus (MuLV) reverse transcriptase (Invitrogen), 0.5 μg of oligo-dT_{12-18}, 4 μL of 5× RT buffer, 0.5 mM dNTP, and 1 U of RNaseout recombinant RNase inhibitor. Q-PCR was performed in triplicate using 25 μL of a reaction mixture containing 12.5 μL of Fast SYBR Green Master Mix (ABI, Applied-Biosystems, Foster City, CA, USA), 50 ng of cDNA, and gene-specific forward and reverse primers at 1 μM final concentration in a thermal cycler (7900HT Fast Real-Time PCR System, ABI). The PCR cycling had an initial incubation at 95 °C for 3 min followed by 45 cycles of denaturation at 95 °C for 15 s and reaction for annealing and extension at 60 °C for 1 min. The PCR primers used for amplification included the following: β-actin (sense, 5′-TCCCTGGAGAAGAGCTACG-3′ and antisense, 5′-GTAGTTTCGTGGATGCCACA-3′), CD66a (sense, 5′-CTCTCCTGCTATGCAGCCTC-3′ and antisense, 5′-ACTGTGGTCTTGCTGGCTTT-3′), CD66b (sense, 5′-GAAACAGTGGATGCCAACCG-3′ and antisense, 5′-GAGTCTCCGGATGTACGCTG-3′), CD66c (sense, 5′-ACCGTCCAGGGGAAAATCTG-3′ and antisense, 5′-AGGAGCACTTCCAGAGACTGT-3′), CD66d (sense, 5′-GACACAAACATTTACTGCCGGA-3′ and antisense, 5′-GAGAGGCCTTTGTCCTGACC-3′), HTR2A (sense, 5′-AGGGTGCCTCTCACCGTCGT-3′ and antisense, 5′-AAGCTTGCTCGGCAGAGGCC-3′), HTR2B (sense, 5′-ACTGCACTGGGCAGCTCTTCTG-3′ and antisense, 5′-GTGGGAGGGGCCACATAGCCT-3′), and HTR2C (sense, 5′-ACGTGCGTGCTCAACGACC-3′ and antisense, 5′-TCGGCGTGCGTTCTGGTCTT-3′). DRD1 (sense, 5′-GGGACTGGGCTGGTGGTGGA-3′ and antisense, 5′-CAGCCACTGCCTTCCAGGGC-3′), DRD2 (sense, 5′-TGTGCACGGCGAGCATCCTG-3′ and antisense, 5′-GACCACGAAGGCCGGGTTGG-3′), DRD3 (sense, 5′-GCCAGGACACTGCCTTGGGTG-3′ and antisense, 5′-CCCGAAGTGGCACTCCCCGA-3′), DRD4 (sense, 5′-CGCCCTCCCCACTCCTTGGT-3′ and antisense, 5′-TGGGCTACGTCAACAGCGCC-3′), DRD5 (sense, 5′-TACCCGGGGCAGTTCGCTCT-3′ and antisense, 5′-CTGCGCACACCAGCACGTTG-3′). The relative expression levels of the target cDNA were calculated after normalizing the relative intensity of target cDNA to the intensity of β-actin.

2.4. Phagocytosis

Laboratory strain *E. coli* DH5α bacteria were grown in LB broth (5 g/L yeast extract, 10 g/L tryptone, 10 g/L NaCl, and 31.24 mg/L 2,2′-dipyridyl). *E. coli* were stained with 5 μM Oregon Green 488-X succinimidyl ester (Molecular Probes, Eugene, OR, USA) and 1 mg/mL human IgG (Sigma) for 30 min in the dark at room temperature. After washing three times to remove excess fluorescent dyes, bacteria were kept on ice until use. Cells and bacteria were prepared and suspended in Na-medium (5.6 mM glucose, 127 mM NaCl, 10.8 mM KCl, 2.4 mM KH_2PO_4, 1.6 mM $MgSO_4$, 10 mM HEPES, 1.8 mM $CaCl_2$, pH 7.3). Phagocytosis of bacteria was performed in Na-medium at 37 °C for 5 min at a bacteria/cell ratio of 2:1. The incubation was stopped by the addition of 2 mL of ice-cold phosphate buffered saline (PBS), and the cells were then washed three times with ice-cold PBS. After fixing the cells with 4% paraformaldehyde, phagocytic uptake was analyzed using a FACS flow cytometer (FACScan, Becton Dickinson, Franklin Lakes, NJ, USA).

2.5. Intracellular survival assays

APD-treated, ATRA-treated HL-60 cells were prepared, and phagocytic uptake of *E. coli* was performed at 37 °C for 1 h at a bacteria/cell ratio of 10:1. The phagocytic process was stopped by placing the samples on ice. Subsequently, gentamicin (final concentration 200 μg/mL) was added to kill extracellular bacteria, and the tubes were incubated for an additional 60 min on ice. The neutrophils were then lysed by incubating the samples for 20 min in 2% saponin. The number of *E. coli* colony-forming units (CFU) was determined.

2.6. Isolation of human neutrophils

Peripheral blood samples were obtained from normal adult donors. Following erythrocyte sedimentation with dextran and lysis of contaminating erythrocytes with buffered potassium chloride, the neutrophils were separated from the leukocyte fraction over a discontinuous gradient of Ficoll-Paque PLUS as previously described [46]. The purity of isolated neutrophils was routinely greater than 95% determined by light microscopy based on nuclear morphology. The purified neutrophils were resuspended in a round-bottom polypropylene tube in RPMI medium supplemented with 10% FBS and antibiotics.

2.7. Measurement of myeloperoxidase (MPO) activity of stimulated neutrophils

Purified human neutrophils were each seeded at 3×10^6 cells/well in 24-well plates and were treated with various APDs for 4 h. Cells were further treated with 1 μM N-Formyl-Met-Leu-Phe (fMLP, Sigma-Aldrich) and 5 μg/mL cytochalasin B (CB, Sigma-Aldrich) for another 30 min. The supernatants of cells were collected and levels of MPO in the supernatants were determined using a neutrophil myeloperoxidase activity assay kit (Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturer's instructions.

2.8. Measurement of IL-8 production of stimulated neutrophils

Purified human neutrophils were each seeded at 3×10^6 cells/well in 24-well plates and treated with various APDs for 4 h. Cells were further