The impact of clozapine on regulation of inflammation in murine macrophage cells

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Abstract

OBJECTIVES: Clozapine is an atypical antipsychotic drug known for its impact on production of pro-inflammatory cytokines. The aim of our work was to examine the impact of clozapine on the production of interleukin 6 (IL-6) by the LPS (lipopolysaccharide) stimulated macrophage cells and to confirm or exclude that it regulates the inflammation due to its interaction with alpha 7 nicotinic receptor (nAChR). Secondly, we focused on a verification of the antioxidant effect of clozapine.

METHODS: The levels of IL-6 in cell culture media were determined by ELISA method. Antioxidant properties of clozapine were estimated based on the reduction of 2,2-diphenyl-1-picrylhydrazyl radical.

RESULTS: The IL-6 level produced by cells treated clozapine decreased significantly from IL-6 level created by clozapine-untreated cells. However, the production of IL-6 in the cells treated with clozapine and simultaneously with selective alpha 7 nAChR antagonist methyllycaconitine did not alter significantly from the IL-6 production in the cells treated just with clozapine. The free radical scavenging activity of clozapine in concentration 1.00 mM was found equivalent to 0.13 mM of standard antioxidant *N*-acetyl-L-cystein.

CONCLUSION: Our study confirmed, that clozapine reduces production of IL-6 in LPS-activated macrophage cells nevertheless we denied that it would be mediated through alpha 7 nAChR. Moreover antioxidant potential of clozapine was observed.

Abbreviations:

IL-6	- interleukin 6
LPS	- lipopolysaccharide
nAChR	- nicotinic acetylcholine receptor
ELISA	- enzyme-linked immunosorbent assay
DPPH	- 2,2-diphenyl-1-picrylhydrazyl radical
MLA	- methyllycaconitine
DMSO	- dimethyl sulfoxide
MTT	- 4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PBS	- phosphate buffered saline
NAC	- *N*-acetyl-L-cystein

ORIGINAL ARTICLE

INTRODUCTION

Clozapine is an atypical antipsychotic drug used for the treatment of schizophrenic patients resistant to the therapy with standard neuroleptic drug (Haack *et al.* 2003). Both positive and negative impact of clozapine on production of several pro-inflammatory cytokines has been reported in schizophrenia patients (Maes *et al.* 1997), lipopolysaccharide (LPS) treated mice (Sugino *et al.* 2009), offspring of LPS-treated mice (Basta-Kaim *et al.* 2012) and LPS-stimulated macrophage cells (Chen *et al.* 2013). Unfortunately, exact mechanism has not been revealed in the quoted papers. For the reason, we decided to get our effort to work with macrophage cell lines and test whether clozapine can be involved here. This would be in a compliance with the quoted papers.

Two systematic quantitative reviews of cross-sectional studies assessing in vivo plasma or serum cytokine concentrations or in vitro secretion of cytokines by peripheral blood leukocytes from schizophrenia patients and healthy volunteers published before the year 2010 was done by Potvin *et al.* (2008) and Miller *et al.* (2011). On the base of the results of these two studies, the interleukin 6 (IL-6) seems to be closely related to schizophrenia.

We hypothesized that the decline in IL-6 levels caused by the clozapine treatment might be explained by the interaction of clozapine with the alpha 7 nAChR on the peripheral macrophage cells and activation of cholinergic anti-inflammatory pathway. The objective of our work was to examine the impact of clozapine on the production of IL-6 by the LPS stimulated murine macrophage cells j774.2 with or without presence of the selective alpha 7 nAChR antagonist methyllycaconitine (MLA) (Panagis etal. 2000; Pohanka 2012). Because of the evidences, that schizophrenia is associated with oxidative stress (Ng *et al.* 2008) and that clozapine has an impact on the oxidative stress signs in social isolation rearing rats` model of schizophrenia (Möller *et al.* 2011), our second aim was to verify whether clozapine can have an antioxidant effect.

MATERIAL AND METHODS

Cell culture

The murine macrophage cell line j774.2 (ATCC, Manassas, USA) was maintained in Dulbecco`s modified Eagle`s medium supplemented with 10% fetal bovine serum, both purchased from PAA (Pasching, Austria), at 37 °C, in a 5% CO_2 atmosphere and saturated with atmospheric moisture.

Cell viability

For the 24 hours, 200 µl of cell suspension (3×10^5 cells/ml) was incubated in each well of a disposable 96-well plate. The cells were treated with clozapine (0, 0.4, 1.2 and 6 µg/ml). After other 24 hours, the cell viability was evaluated using standard MTT assay. MTT dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was purchased from Sigma Aldrich, Saint Louis, USA. After the removing of medium, 100 µl of MTT dye solution (5 mg/ml in phosphate buffered saline; PBS) was added into each well and the cells were incubated at 37 °C for 90 min. Then the MTT solution was removed and 100 µl of dimethyl sulfoxide (DMSO, Sigma-Aldrich) was added. The plate was gently stirred and the absorbance at 570 and 630 nm was read using Synergy 2 Multi-Mode Microplate Reader (BioTek Instruments, Inc., Vermont, USA).

LPS stimulation and exposure of clozapine and MLA

The cells pre-incubated for 24 hours in a 96-well plate (3×10^5 cells/ml) were treated with 1 µg/ml of LPS from *Escherichia coli* (Sigma Aldrich) and simultaneously with 0, 0.4, 1.2 and 6 µg/ml of clozapine (Sigma Aldrich) or with 6 µg/ml of clozapine and 10^{-11}–10^{-5} M MLA (Sigma Aldrich) except the untreated control cells. After the 24-hour-incubation the medium was collected and centrifuged at 250×*g* and 25 °C for 10 minutes to remove debris. Then it was stored at –70 °C until the IL-6 levels were assessed. Each experiment was performed in three triplicates.

IL-6 assay

The levels of IL-6 in cell culture media were determined by ELISA using Murine IL-6 Elipair kit (Abcam, Cambridge, UK). The samples of the cell culture media were tenfold diluted in PBS containing 1% of bovine serum albumin (Sigma Aldrich) before the ELISA. An antibody specific for murine IL-6 was coated onto the wells of the standard ELISA plates. Samples and standards of known murine IL-6 concentrations were pipetted into these wells. A biotinylated antibody specific for murine IL-6 was added. Then, a streptavidin-peroxidase conjugate was added as well. After overnight incubation and washing by PBS, a solution of 3,3',5,5'-tetramethylbenzidine was used to induce a colored reaction product. The intensity of this colored product was measured at 450 nm as the primary wavelength and 620 nm as the reference wave length using ELISA reader Sunrise (Tecan, Männedorf, Austria).

Antioxidant activity of clozapine

Antioxidant properties of clozapine were estimated using DPPH test based on the reduction of violet 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) to the yellow 2,2-diphenyl-1-picrylhydrazin. The method was slightly modified from referred protocol (Kedare & Singh 2011). *N*-acetyl-L-cystein (NAC) was purchased in an analytical purity (Sigma Aldrich, Saint Louis, USA). The substance was used as a standard antioxidant, was diluted in PBS to $10^{-5.5}$–10^{-3} M standard solutions. Clozapine was dissolved in DMSO to concentration 4.8 µg/ml. The stock solution was diluted to final concentrations 10^{-6}–10^{-3} M in PBS. 500 µl of NAC or clozapine solution was mixed with 500 µl of 0.2 mM

solution of DPPH in methanol. After the color stabilization, absorbance of the mixture was measured at 517 nm. The antioxidant ability of clozapine was compared to NAC using the calibration curve.

Statistical analysis

Due to a relatively small sample sizes and fact, that data did not follow the demand of normality, comparison of the obtained results was done using non-parametric Kruskal-Wallis test with multiple comparisons. Statistical analysis was performed on Statistica software'98 Edition (StatSoft, Tulsa, USA). The calibration curve for DPPH assay was constructed using the software Origin 8 SR2 (OriginLab Corporation, Northampton, USA).

RESULTS

Cell viability

After the 24-hour-lasting incubation, no significant difference between viability of the untreated cells and the cells treated with clozapine was found by MMT assay. The result is true even for the upper concentrations of clozapine.

Impact of LPS, clozapine and MLA exposure on the IL-6 production

The ELISA method used to determine the production of IL-6 was linear in the concentration range from 7.80 to 500 pg/ml. LPS widely stimulated the inflammatory response of the cells as the level of IL-6 produced by all stimulated cells was about 1,000 times higher than in the unstimulated cells. In control represented by the LPS-untreated cells, we did not observe any alteration in the IL-6 levels caused by clozapine which indicates no inflammatory reaction. As it is displayed in the Figure 1, IL-6 produced by the stimulated cells treated with clozapine descended in a dose-dependent manner. In the cells treated with the lowest used clozapine concentration 0.4 μg/ml, the insignificant decrease in clozapine production was observed (from 21.1±2.9 μg/ml to 15.3±2.3 μg/ml). The IL-6 level produced by cells treated with 6.0 μg/ml and 12 μg/ml of clozapine differed significantly (p=0.01 resp. p<0.001) from IL-6 level created by clozapine-untreated cells. However, the production of IL-6 in the cells treated with clozapine and simultaneously with 10^{-11}–10^{-5} M MLA remains quite stable and did not alter significantly from the IL-6 production in the cells treated just with clozapine. The results are shown in Figure 2.

Antioxidant activity of clozapine

The free radical scavenging activity of clozapine in concentration 0.01 mM was found to be equivalent to the antioxidant activity of 0.02 mM NAC, clozapine in concentration 0.10 mM was equivalent to 0.08 mM of NAC and clozapine in concentration 1.00 mM was equivalent to 0.13 mM of NAC.

DISCUSSION

In human schizophrenic patient therapy, the recommended threshold plasma level of 0.30–0.42 μg/ml of clozapine is associated with an increased probability of good clinical response. The toxic effect of clozapine can occur when the plasma level exceeds 1 μg/ml (Haack *et al.* 2003). Several studies suggest that a minimum effective clozapine plasma concentration starts on the level of 0.35 μg/ml (Perry *et al.* 1998; Buur-Rasmussen & Brøsen 1999). The upper limit of the therapeutic interval is quite unclearly defined. The risk of central nervous system side-effects seems to increase with

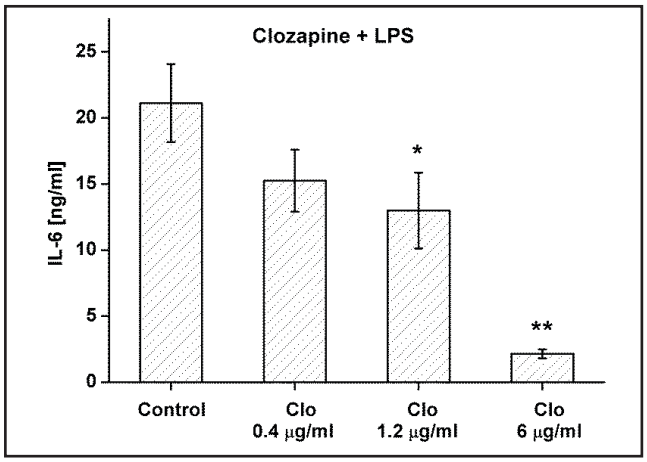

Fig. 1. The release of IL-6 in LPS-stimulated cells treated by clozapine (Clo), experiment in triplicate. Significant decrease was found in cells treated with 1.2 μg/ml of clozapine (*p=0.01) and 6 μg/ml of clozapine (**p<0.001).

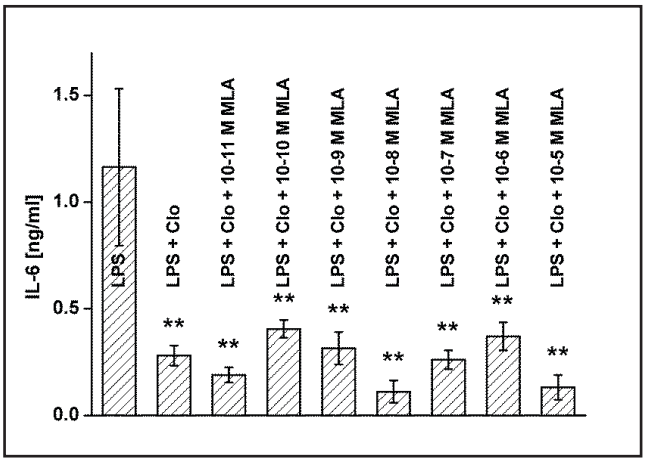

Fig. 2. The release of IL-6 in LPS-stimulated cells treated by 6 μg/ml of clozapine in presence of MLA in concentration 10^{-11}–10^{-5} M, experiment in triplicate. The IL-6 release declined significantly in all clozapine-treated cells (**p<0.001) no significant difference between the cells treated with clozapine alone and the cells treated with clozapine and MLA.

concentrations above 1.3 μg/ml and the occurrence of agranulocytosis, the most serious side-effect of clozapine treatment, does not seem to be dose-related (Buur-Rasmussen & Brøsen 1999). In the presented experiment, we used clozapine in a concentration range corresponding to the reported plasma therapeutic levels (0.4 μg/ml) and even higher (1.2 μg/ml and 6 μg/ml). As the results of MTT assay did not show any decrease of cell viability caused by clozapine treatment, we can conclude, that decline of the IL-6 production was not due to suppression of the cells growth.

Considering our results, clozapine was proved to be able to suppress production of pro-inflammatory cytokine IL-6 in LPS-activated macrophage cells. This finding is in compliance with the papers pointed on pro-inflammatory cytokine production in LPS-treated mice (Sugino *et al.* 2009), monocyte-derived macrophages (Chen *et al.* 2013) and in the schizophrenia model of the offspring of LPS-treated rats (Basta-Kaim 2012) as well. On the other hand, preponderance of data seems to point to an increase of IL-6 levels as a result of clozapine application (Haack *et al.* 2003; Maes *et al.* 1997; Røge *et al.* 2012; Contreras-Shannon 2013). We concluded that clozapine causes decline of IL-6 level when the monocyte-macrophage arm of immune system is stimulated by LPS. On the other hand, clozapine increases the level of IL-6 when adaptive immunity is activated.

Stimulation of alpha 7 nAChR on macrophages leads to inhibition of TNFα (Tracey 2002; Wang *et al.* 2003) and IL-6 (Xue *et al.* 2014; Waldburger *et al.* 2008) excretion. Inferring the fact that clozapine treatment is related to the alpha 7 nAChR stimulation (Stevens *et al.* 2013), modulation of immunity via 7 nAChR can be expected as well (Pohanka 2012). MLA is known to be a selective antagonist of alpha 7 nAChR (Panagis *et al.* 2000; Waldburger *et al.* 2008). For this reason, if MLA impacted against the clozapine's IL-6 suppressive effect, we could infer, that clozapine would acted due to the alpha 7 nAChR. Unfortunately, no impact of MLA on the IL-6 levels decreased by clozapine was proved in our experiment. Owing to these findings, it can be concluded, that clozapine`s anti-inflammatory properties on activated macrophages are not due to its agonist effect on the alpha 7 nAChR.

As it can be found in published papers, clozapine is able to influence a number of different receptors in the brain, mainly receptors coupled with G protein (Schmid *et al.* 2014). Among neural mediators, dopamine seems to play a significantrole in immune regulation (Basu *et al.* 1993). Macrophages express dopamine receptors like D3 dopamine receptor (Gupta *et al.* 2011). Other mediators, function of which can be altered by clozapine, may influence immune processes in macrophages as well. For instance, serotonin was found to reduce the initial LPS induced nitric oxide burst and to suppress LPS induced cytokine (IL-1) production (Tucci *et al.* 2013). It is an interesting finding because macrophages are known to express different types of serotonin receptors (de las Casas-Engel *et al.* 2013). Because of these facts, it is obvious, that effect of clozapine embraces various receptors and several potential pathways. Most of the pathway can cause immunomodulation. Further investigation is needed to explain the exact mechanism prior to identify exact mechanism of clozapine action on immunity.

In the present study, the free radical scavenging activity of clozapine was investigated by DPPH test. The value corresponds to an antioxidant potential of a compound or mixture (Möller *et al.* 2011; Kedare & Singh 2011). The free radical scavenging activity of clozapine is quite comparable to the scavenging activity of NAC. This finding is in compliance with the work published by Möller *et al.* (Ng *et al.* 2008; Möller *et al.* 2013) and gives an explanation of the described improvement of oxidative damage in social isolation rearing rats`model of schizophrenia after the clozapine administration (Möller *et al.* 2011). Moreover, similar therapeutic effect of clozapine and NAC in the model animals was revealed (Möller *et al.* 2013).On the other hand, Ribeiro *et al.* (2013) described no normalization of oxidative stress markers like reduced glutathione levels and lipid peroxidation in the rats exposed in a neonatal age to the viral mimetic polyI:C. Clozapine nevertheless reversed microglial activation and inducible nitric oxide synthase increase.

CONCLUSIONS

In a summary, our study confirmed, that clozapine reduces production of IL-6 in LPS-activated macrophage cells. This is true even despite fact that we denied that it would be mediated through alpha 7 nAChR. The role of other receptors expressed by macrophages, for example dopamine or serotonin receptors, is discussed and suggested for further examination. Moreover antioxidant potential of clozapine was observed. The fact contributes to the therapeutic efficacy of clozapine.

Conflict of interests

I have no financial interest in this manuscript and no affiliations (relationships) to disclose

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