The effects of clozapine on the GSK-3-mediated signaling pathway

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Abstract We investigated the effect of 10 μM clozapine on the activity of glycogen synthase kinase-3β (GSK-3β) and its upstream and downstream molecules in SH-SY5Y human neuroblastoma cells. Clozapine activates both Akt- and Dvl-mediated phosphorylation of GSK-3β through phosphorylation at Ser9, and increased total cellular and intranuclear levels of β-catenin. Pretreatment with the specific inhibitor of the phosphatidylinositol 3-kinase (PI3K)-Akt pathway, LY294002 (20 μM), prevented the phosphorylation of Akt but did not affect the phosphorylation of GSK-3β. These results suggest that clozapine regulates the phosphorylation of GSK-3β through Wnt signal pathways involving Dvl upstream but not through the PI3K-Akt pathway in SH-SY5Y cells.

Key words: Clozapine; Glycogen synthase kinase-3β; Dvl; Wnt; Phosphatidylinositol 3-kinase

1. Introduction

Clozapine is a representative of 'atypical' antipsychotic agents and its major indication is treatment-resistant schizophrenia [1,2]. However, it can also be successfully used for the treatment of treatment-resistant bipolar disorder [1] and it is proposed that clozapine also has considerable mood-stabilizing effects [3]. Glycogen synthase kinase-3 (GSK-3) is commonly affected by different kinds of mood stabilizers such as lithium and valproate. The inhibition of GSK-3 by these drugs could be responsible for their neuroprotective properties or their therapeutic effects [4–6].

GSK-3 works in two major contexts [7]. The one is receptor tyrosine kinase-mediated signaling. In this context, the uppermost signaling event is receptor binding of growth factors like insulin. Thus, autophosphorylated receptor activates the phosphatidylinositol 3-kinase (PI3K), and the cascade of signaling event leading to the activation of protein kinase B (Akt/PKB). Akt phosphorylates GSK-3 and GSK-3 phosphorylated on its Ser9 residue loses its enzymatic activity [8]. This results in the decreased phosphorylation of its substrate, glycogen synthase. Glycogen synthase is subsequently activated as a result of the decreased phosphorylation.

The other major context of GSK-3 signaling is mediated via the seven transmembrane receptor Frizzled, and the extracellular signal Wnt. Wnt-mediated GSK-3 signaling is completely different from that mediated by insulin. In this system, GSK-3 works within a multimolecular complex composed of several molecules including Axin, APC, and β-catenin [9,10]. Without upstream signal, dephosphorylated GSK-3 is catalytically active and phosphorylates its downstream molecule β-catenin. When Wnt binds to Frizzled, the adapter protein Dishevelled (Dvl) is phosphorylated, and this leads to the phosphorylation-deactivation of GSK-3 [11]. Through this deactivation, the phosphorylation status of β-catenin is decreased, which results in release of β-catenin from the complex. The released β-catenin enters into the nucleus, where it binds to the T-cell factor family transcription factor (also known as the lymphoid-enhancer factor family) and induces the transcription of target genes [12].

In this study, we examined the effect of clozapine on the signaling cascade related to GSK-3. Since other mood-stabilizing agents such as lithium and valproate have similar effects on GSK-3 [4,5], we speculated that clozapine may also affect the signaling system mediated by GSK-3 in a similar fashion.

2. Materials and methods

2.1. Cell culture, serum starvation and drug treatment

The human neuroblastoma cell line SH-SY5Y (ATCC) was grown in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) supplemented with 10% (v/v) fetal bovine serum and 1% penicillin–streptomycin (Gibco BRL) in a 37°C humidified incubator with 5% CO_2. For serum starvation, cells cultured in the complete growth medium were changed to serum-free DMEM and incubated for 24 h. Clozapine (Tocris) was dissolved in dimethylsulfoxide (DMSO) at a concentration of 100 μM. To block the activity of PI3K, the cells were pretreated with LY294002 (Sigma) at a concentration of 20 μM, 1 h before clozapine treatment. For in vitro dephosphorylation of protein, lysates were treated with 50 U of alkaline phosphatase (Sigma) at 30°C for 30 min and diluted with an equal volume of cell lysis buffer.

2.2. Electrophoresis and Western blotting

Cells were washed in ice-cold phosphate-buffered saline (PBS) and lysed in RIPA(+) (0.1% sodium dodecyl sulfate (SDS), 1.0% Triton X-100 and 1.0% deoxycholate in PBS) containing 1 mM dithiothreitol (DTT), 1% protease inhibitor cocktail (Sigma), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM Na_3VO_4, 1 mM NaF and 1 mM β-glycerophosphate at 4°C for 15 min. Proteins were separated by SDS–polyacrylamide gel electrophoresis (PAGE). Immunoblotting with primary antibodies including anti-phospho-GSK-3β (Ser9), anti-GSK-

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Abbreviations: GSK-3β, glycogen synthase kinase-3β; Dvl, dishevelled; PI3K, phosphatidylinositol 3-kinase

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3β, anti-phospho-Akt, anti-Akt, anti-phospho-β-catenin (Ser33/37/Thr41) (Cell Signaling Technology), anti-phospho-GSK-3β (Tyr216) (Upstate Biotechnology), anti-poly (ADP-ribose) polymerase (PARP), anti-caspase-3 (Santa Cruz) and anti-β-catenin (BD Transduction Laboratories) was done. Immunoblotting was performed as described [13].

2.3. Preparation of nuclear extract

SH-SY5Y cells were washed with PBS three times, resuspended in 500 μl of ice-cold buffer A (10 mM HEPES-KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF) and incubated on ice for 15 min. Then 0.1% Nonidet P-40 (NP-40) was added to the cell extract, incubated on ice for 5 min and centrifuged at 12 000 rpm for 30 s at 4°C. Nuclear proteins were extracted by addition of 100 μl of buffer B (20 mM HEPES, pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, protease inhibitor cocktail) for 30 min at 4°C with occasional vortexing. After centrifugation at 13 000 rpm for 5 min at 4°C, supernatants were collected and stored at −70°C.

2.4. Immunofluorescence staining

SH-SY5Y cells were grown on round coverslips in multiwell culture plates and exposed to clozapine (10 μM) for indicated times. For β-catenin staining, the permeabilized cells were incubated with anti-β-catenin antibody (BD Transduction Laboratories) for 3 h at room temperature. After washing, the cells were stained with fluorescein isothiocyanate (FITC)-conjugated secondary antibody for 1 h and mounted on slide glasses with mounting medium.

2.5. DAPI staining

SH-SY5Y cells grown as described above were exposed to clozapine for the indicated times. The resulting cells were stained with 4′,6′-diamidino-2-phenylindole (DAPI) (0.4 μl/ml; Sigma) for 5 min, washed, and then examined with a fluorescence microscope. Apoptotic cells were scored on the basis of the presence of highly condensed or fragmented nuclei.

2.6. MTT assay

SH-SY5Y cells were seeded in a 96-well tissue culture plate and incubated for 24 h. After treatment with clozapine, cells were incubated in yellow 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) solution (0.5 mg/ml). After incubation, the medium containing MTT solution was removed and each well received 100 μl DMSO that solubilized the preformed purple formazan salt crystals. The solubilized formazan product was spectrophotometrically quantified using an ELISA reader.

2.7. Analysis of apoptotic cell population by FACS

Exponentially growing cells were treated with clozapine, collected, and fixed with chilled 70% EtOH. Ten thousand cells stained with propidium iodide (PI) were analyzed on a fluorescence-activated cell sorter (FACSCalibur; Becton Dickinson), and the resulting DNA histograms were converted to proportions of each cell cycle phase by the ModiFit LT software (Becton Dickinson).

3. Results

3.1. Effect of clozapine on cell survival

We observed the effect of clozapine on cell survival and death. When we treated the cells with increasing concentrations of clozapine, cell death occurred at the concentration of 30 μM or more (Fig. 1A). The doubling time was also somewhat retarded with concentrations of 30 μM or more of clozapine (data not shown) and ensuing experiments were done using 10 μM of clozapine. There were no changes (Fig. 1B) in the cellular morphology and DAPI staining also showed intact nuclei until 10 μM clozapine (Fig. 1C). There was also no cleavage of PARP and pro-caspase-3 (Fig. 1D), indicating no apoptosis at this concentration. FACS results also showed that there was no increase in the fragmented DNA (Fig. 1E). In contrast, when the cells were exposed to a pro-apoptotic control stimulus (heat shock up to 45°C for 90 min) and observed 48 h later, the results were positive for all apoptotic screening tests.

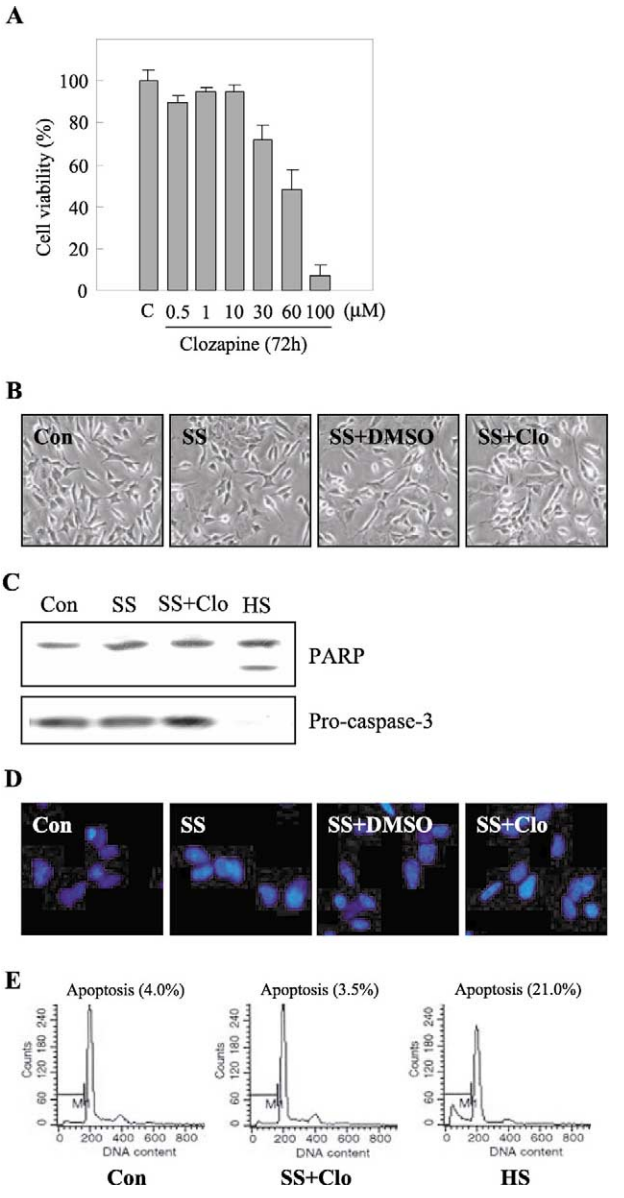

Fig. 1. A: SH-SY5Y cells were treated with 1–100 μM clozapine for 72 h and cell viability was measured by the MTT assay. Data are shown as the means of triplicate samples and represent the viability of cells compared with untreated cells. B: Serum-starved (SS) SH-SY5Y cells were exposed to 10 μM clozapine (Clo) for 24 h. Original magnification 200×. C: Serum-starved SH-SY5Y cells exposed to either 10 μM clozapine for 24 h or heat shock (HS) at 45°C for 90 min and recovered at 37°C for 48 h and the cellular proteins were analyzed by SDS–PAGE and Western blotting with anti-PARP and anti-pro-caspase-3. D: Serum-starved SH-SY5Y cells were exposed to 10 μM clozapine for 24 h. The cells were stained with DAPI and the nuclei were observed under a fluorescence microscope. E: Serum-starved SH-SY5Y cells were exposed to 10 μM clozapine or heat shock for the indicated times and stained with PI and analyzed by FACS analysis.

3.2. Effect of clozapine on GSK-3β phosphorylation

GSK-3β was constitutively phosphorylated below 10% serum content. So we reduced the concentration of serum to

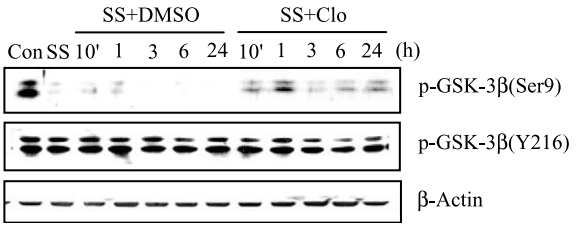

Fig. 2. Serum-starved (SS) SH-SY5Y cells were exposed to 10 μM clozapine (Clo) for the indicated times and the cellular proteins were analyzed by SDS–PAGE and Western blotting with anti-phospho-GSK-3β (Ser9, Tyr216), and anti-β-actin antibodies. In certain conditions, the phospho-GSK-3β antibody detected two separate bands. According to the manufacturer's guidelines, the upper band is probably phosphorylated at another site of GSK-3β as well.

0%, i.e. serum-free medium (referred to as 'serum starvation' in this article) to single out the effect of clozapine. Despite this low serum concentration, there was no remarkable cell death and this condition passed the entire apoptotic screening test described above, until the very last time point observed. Under serum-starved condition, the phosphorylation of GSK-3β was reduced compared with 10% serum condition. Adding clozapine (10 μM) to this serum-starved condition increased the phosphorylation of GSK-3β within 10 min. And the phosphorylation was maintained over 24 h (Fig. 2).

3.3. Upstream event of GSK-3β phosphorylation

The phosphorylation of GSK-3β can be regulated by two major upstream pathways. One is the Akt pathway and the other is the Wnt signaling pathway mediated by increased phosphorylation of the adapter protein, Dvl. Using specific antibody against phosphorylated Akt, we demonstrated that Akt is also phosphorylated by clozapine at similar time points as the phosphorylation of GSK-3β (Fig. 3A). The phosphorylation of Dvl was also demonstrated using band mobility shift in Western blot analysis (Fig. 3B). After treatment of the cell lysate with alkaline phosphatase, the shifted Dvl band disappeared along with the phosphorylated band of GSK-3β and Akt. Since a specific inhibitor of the PI3K-Akt pathway was available, we pretreated the cells with LY294002 (20 μM) and then with clozapine. As expected, LY294002 pretreatment abolished the phosphorylation of Akt. However,

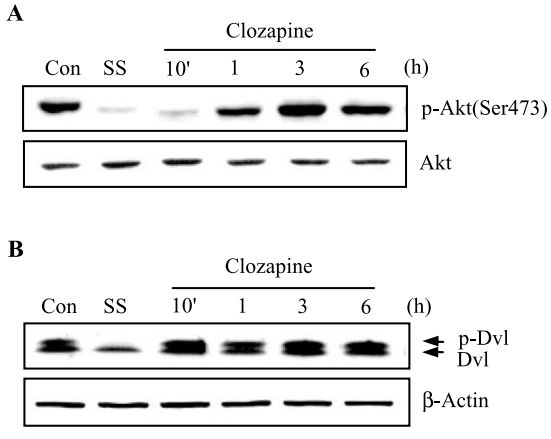

Fig. 3. Serum-starved (SS) SH-SY5Y cells were exposed to 10 μM clozapine (Clo) for the indicated times and the cellular proteins were analyzed by SDS–PAGE and Western blotting with anti-phospho-Akt (A) and anti-Dvl (B).

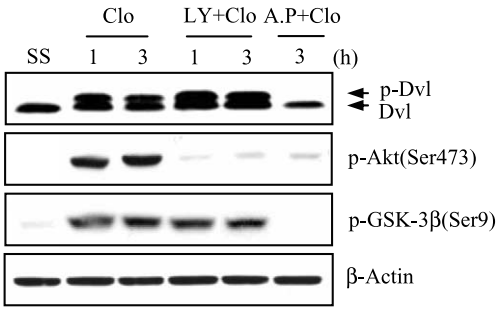

Fig. 4. Serum-starved (SS) SH-SY5Y cells were pretreated with 20 μM LY294002 (LY) for 1 h and exposed to 10 μM clozapine (Clo) for 1 h and 3 h in the presence of the inhibitors and the cellular proteins were analyzed by SDS–PAGE and Western blotting with antibodies to anti-phospho-GSK-3β, anti-phospho-Akt, and anti-Dvl. Serum-starved SH-SY5Y cells were exposed to 10 μM clozapine for 3 h, and extracts lysates were treated with 50 U of alkaline phosphatase (A.P.) (Sigma) at 30°C for 30 min, and the cellular proteins were analyzed by SDS–PAGE and Western blotting with antibodies to phospho-GSK-3β, phospho-Akt and Dvl.

the phosphorylation of GSK-3β was not changed by LY294002 pretreatment (Fig. 4). This suggested indirectly that the Wnt pathway is more critical for GSK-3β phosphorylation under the clozapine paradigm.

3.4. Downstream pathway of GSK-3β

Since the Wnt pathway seemed more critical, we observed the downstream pathway associated with Wnt signaling, i.e. β-catenin. First, we examined changes in β-catenin phosphorylation in response to clozapine. Treatment with clozapine resulted in accumulation of β-catenin through dephosphorylation at Ser33/37/Thr41 (Fig. 5A). Next, we observed the changes in the amount of β-catenin. Clozapine treatment increased the amount of β-catenin (Fig. 5B). We fractionated the cell lysate into nuclear and cytoplasmic portions, and determined the amount of β-catenin separately. There was clear evidence that the nuclear content of β-catenin was selectively increased (Fig. 5B). Then we demonstrated this nuclear migration by immunofluorescence staining. As shown in Fig. 5C, under the control condition, the β-catenin was concentrated at the cell–cell junction. There was also diffuse cytoplasmic staining, save the nucleus. When the cells were treated with clozapine, the number of cells showing nuclear staining of β-catenin was increased. In conclusion, clozapine treatment increased the amount of β-catenin, and induced its migration into the nucleus.

4. Discussion

In these experiments, we found that clozapine is not cytotoxic up to a concentration of 10 μM, and may even have neurotrophic effects like the growth factors. In the serum-starved cells the phosphorylations of Akt and GSK-3β were reduced compared to the serum-stimulated condition. Clozapine treatment restored the phosphorylation status of Akt and its potential downstream molecule GSK-3β. These effects of clozapine on Akt and GSK-3 are also achieved by another mood-stabilizing agent, valproate, whereas lithium, another mood stabilizer, does not phosphorylate Akt but still phosphorylates GSK-3 [5]. Since GSK-3 can be phosphorylated by multiple kinases, the upstream event may be different with

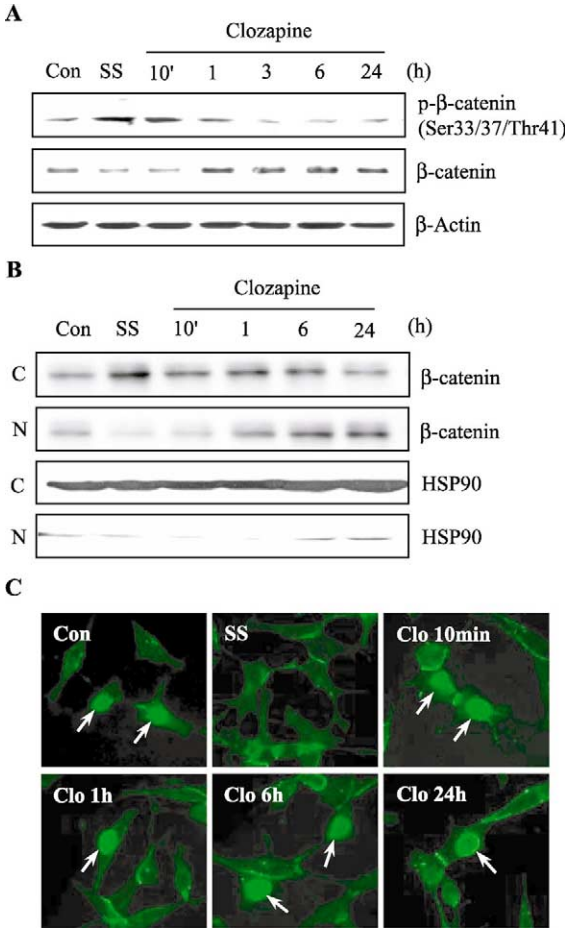

Fig. 5. A: Serum-starved (SS) SH-SY5Y cells were exposed to 10 μM clozapine (Clo) for the indicated times and the cellular proteins were analyzed by SDS–PAGE and Western blotting with antibodies to anti-phospho-β-catenin (Ser33/37/Thr41), anti-β-catenin and anti-β-actin. B: Serum-starved SH-SY5Y cells were exposed to 10 μM clozapine for the indicated times. Nuclear extracts (N) and cytoplasmic extracts (C) were analyzed by SDS–PAGE and Western blotting with antibodies to β-catenin and HSP90. HSP90 is not regulated in our experimental conditions, and was used as a loading control. C: Serum-starved SH-SY5Y cells were exposed to 10 μM clozapine for the indicated times. The cells were stained with anti-β-catenin antibody and FITC-conjugated secondary antibody and observed under a fluorescence microscope.

different stimuli [14,15]. In cerebellar granule cells, it was reported that lithium does phosphorylate Akt [16]. So, differences in the systems should also be considered. Although the upstream event of Akt phosphorylation was not elucidated, the effect of clozapine is thought to be mediated by PI3K activation since a PI3K inhibitor abolished Akt activation, just as is the case with valproate. This activation of Akt and GSK-3 demonstrates that there may be similarities in the mechanism of action between valproate and clozapine.

However, when the phosphorylation-activation of Akt was blocked, the phosphorylation of GSK-3 was not affected. This suggested the activation of another Akt-independent mechanism for GSK-3 phosphorylation with clozapine treatment. In this aspect, clozapine showed similarity to lithium. One possible explanation was the activation of Wnt-mediated signaling [17]. And indeed, this seems to be the case, since the phosphorylation of Dvl [11] was increased after clozapine treatment. The mobility-shifted signal appears to be the phosphorylated Dvl band since in vitro treatment of cell culture lysates with alkaline phosphatase abolished the shifted signal, along with other phospho-protein signals. Although treatment with a specific inhibitor in this pathway was not available, the hypothesis that the activation of Wnt-mediated signaling may increase the phosphorylation of GSK-3 was also supported by the fact that the downstream molecule in this pathway was affected by clozapine treatment. Clozapine increased the amount of β-catenin in whole cell lysates. Dephosphorylation of GSK-3 leads to phosphorylation of β-catenin [18], after which the phosphorylated β-catenin is degraded. Therefore, the reduction of GSK-3 activity by Ser9 phosphorylation in this pathway results in the increase in β-catenin. Moreover, as a transcription factor, β-catenin is mobilized into the nucleus by Wnt signaling. Nuclear migration of β-catenin was evident after clozapine treatment. These data clearly show that clozapine activates the Dvl-mediated GSK-3 phosphorylation. The role of Wnt-mediated signaling in the mature organism is not clear, but may also be related to cell survival just as the PI3K-Akt pathway [19]. So clozapine has dual action on GSK-3 phosphorylation. First it activates Akt, probably via the activation of PI3K. This pathway was similar to serum- or growth factor-mediated signaling. Second, it activates Dvl, probably via the Wnt-Frizzled pathway. It is not certain which one is more important. However, it should be noted that the blocking of the PI3K-Akt pathway did not influence the survival of cells. We have demonstrated that the Wnt pathway transcription factor β-catenin is regulated by clozapine. NF-κB, a representative survival factor activated by the PI3K-Akt pathway, has also been reported to be activated by Wnt signaling in PC12 cells [20].

In conclusion, clozapine has neurotrophic action and activates both Akt-mediated and Dvl-mediated signaling. But the latter seems to have more important effects on the phosphorylation of GSK-3. Clozapine also increased the total cellular and intranuclear level of β-catenin, at concentrations not much higher than the therapeutic concentration. It is necessary to further investigate whether this effect is specific to clozapine or also applies to other antipsychotics and neurotrophic medications.

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