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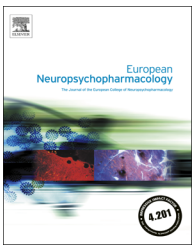

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Insight into mechanism of in vitro insulin secretion increase induced by antipsychotic clozapine: Role of FOXA1 and mitochondrial citrate carrier

A. Menga[a], V. Infantino[d], F. Iacobazzi[a], P. Convertini[a], F. Palmieri[a,b,c], V. Iacobazzi[a,b,c,*]

[a]Department of Biosciences, Biotechnology and Pharmacological Sciences, University of Bari, Via Orabona 4, 70125 Bari, Italy
[b]Center of Excellence in Comparative Genomics, University of Bari, via Orabona 4, 70125 Bari, Italy
[c]CNR Institute of Biomembranes and Bioenergetics, Bari, Italy
[d]Department of Sciences, University of Basilicata, 85100 Potenza, Italy

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Abstract
The use of clozapine and other antipsychotic drugs is known to be associated with a number of adverse metabolic side effects, including diabetes mellitus. These side effects could be, at least in part, the result of impaired islet cell function and abnormal insulin secretion, although the underlying mechanisms are unknown. The aim of this study is the identification of targets for clozapine related to the abnormal insulin secretion. We identify a specific activation of the transcriptional factor FOXA1, but not FOXA2 and FOXA3, by clozapine in HepG2 cells. Clozapine enhances FOXA1 DNA-binding and its transcriptional activity, increasing mitochondrial citrate carrier gene expression, which contains a FOXA1 site in its promoter. Haloperidol, a conventional antipsychotic drug, does not determine any increase of FOXA1 gene expression. We also demonstrate that clozapine upregulates FOXA1 and CIC gene expression in INS-1 cells only at basal glucose concentration.
In addition, we find that abnormal insulin secretion in basal glucose conditions could be completely abolished by FOXA1 silencing in INS-1 cells treated with clozapine.
The identification of FOXA1 as a novel target for clozapine may shed more light to understand molecular mechanism of abnormal insulin secretion during clozapine treatment.

1. Introduction

Clozapine is the prototype of atypical antipsychotic drugs and has been described as a drug of superior efficacy in

*Corresponding author at: Department of Biosciences, Biotechnology and Pharmacological Sciences, University of Bari, Via Orabona 4, 70125 Bari, Italy. Tel.: +39 80 5442322; fax +39 80 5442770.
E-mail address: vito.iacobazzi@uniba.it (V. Iacobazzi).

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otherwise treatment-resistant schizophrenia (Conley et al., 1999; Freedman, 2003). However, subjects treated with clozapine show a high risk of developing different metabolic side effects, such as dyslipidemia, body weight gain, glucose homeostasis disturbance, abnormal insulin secretion and diabetes as compared to the general population (Jin et al., 2004; Raeder et al., 2006; Scheen and De Hert, 2007; Vestri et al., 2007; Kessing et al., 2010). Since insulin regulates lipid and glucose metabolism, the effect of clozapine on insulin secretion and/or on insulin action, at least in part, might explain its capability to induce metabolic disturbances. Regarding the insulin secretion during clozapine treatment, it is unknown whether the insulin levels are increased as secondary effect to drug-induced peripheral resistance or due to a direct effect of the agent on the pancreatic β-cells, or caused by both actions (Yazici et al., 1998; Melkersson et al., 1999). A naturalistic study of clozapine treatment reported that 30–40% of patients received a diagnosis of type II diabetes during the 5- and 10-year follow-ups (Henderson et al., 2000, 2005).

However, despite the number of clinical reports, the molecular mechanism by which clozapine or other antipsychotic drugs, although to a different extent, could cause dysregulation of insulin release and diabetes is still unknown. In general, molecular basis of onset of the metabolic disturbance has been poorly investigated. Only clozapine-induced activation of the sterol regulatory element-binding protein (SREBP) transcription factors related to cellular lipogenesis has been reported (Fernø et al., 2006, 2009). Therefore, it is likely that different genes and related transcriptional factors, important in lipid and carbohydrate homeostasis, in insulin secretion and in β-cells function, might be affected by clozapine treatment. For example, metabolic and secretory features of β-cell are maintained by FOXA1 and FOXA2 (Gao et al., 2010). Members of the winged helix/forkheadbox (FOX) transcription factors subfamily, FOXA1, FOXA2 and FOXA3, play important roles in both metabolism and homeostasis through the regulation of multiple target genes in the liver, pancreas and adipose tissue (Friedman and Kaestner, 2006). FOXA2 mediates fasting responses, including fatty acid oxidation, ketogenesis, gluconeogenesis and increased lipoprotein secretion (Wolfrum et al., 2004; Friedman and Kaestner, 2006; Gao et al., 2010; Convertini et al., 2011). FOXA3 also regulates glucose homeostasis during prolonged fast through maintenance of GLUT2 and gluconeogenic gene expression (Shen et al., 2001). FOXA1 induces glucagon gene expression and insulin secretion in pancreatic cells (Shih et al., 1999; Vatamaniuk et al., 2006). Moreover, FOXA1 plays an important role in regulation of mitochondrial citrate carrier gene expression (CIC) and in insulin secretion (Iacobazzi et al., 2009a).

CIC is an integral inner mitochondrial membrane protein belonging to the mitochondrial carrier protein family SLC25 (Palmieri, 2004, 2012) that catalyzes the export of citrate from the mitochondrial matrix in exchange for cytosolic malate (Bisaccia et al., 1989, 1990). This transporter is essential for fatty acid biosynthesis because citrate in the cytosol is cleaved to acetyl-CoA and oxaloacetate by citrate lyase. Acetyl-CoA is directly used for fatty acid synthesis, and oxaloacetate produces NADPH plus H+ (also necessary for fatty acid production) via malate dehydrogenase and malic enzyme (Kaplan, 2001; Palmieri, 2004). Epigenetic mechanisms and various transcriptional factors regulate human CIC gene expression (Infantino et al., 2007, 2011a; Iacobazzi et al., 2008, 2009a, 2009b). Furthermore, CIC has been found to be involved in the control of glucose-stimulated insulin secretion (Joseph et al., 2006).

This study investigates and identifies transcriptional factors and related genes, particularly involved in insulin secretion, affected by clozapine treatment. We demonstrate for the first time that clozapine, but not haloperidol, specifically upregulates FOXA1 and its regulated CIC gene expression and that FOXA1 silencing completely abolishes the abnormal insulin secretion in INS-1 cells.

2. Experimental procedures

2.1. Construction of plasmids

For heterologous promoter expression, a threefold repeat FOXA site (5′-CCTGGACAATATTTATTTTTGCTG-3′) was cloned into the pGL3 promoter-LUC vector (Promega) upstream of the SV40 basal promoter (Iacobazzi et al., 2009a). For lentivirus-based RNAi experiments, the rat FOXA1 mRNA sequence CTAACCCTTTGCTTGAAAT from 2098 to 2116 bp (Accession no. NM_012742.1) was selected as a hairpin-loop structure by using the siRNA design tool (〈http://jura.wi.mit.edu/bioc/siRNAext/home.php〉). This sequence was cloned in the lenti virus RNAi transfer plasmid pLKO.1 (Sigma) to obtain the pLKO.1-FOXA1 lentivirus silencing construct (Iacobazzi et al., 2009a).

2.2. Cell culture, RNA interference, and transient transfection

INS-1 cells (gift from Dr. P. Maechler) were grown in RPMI 1640 medium (Roswell Park Memorial Institute) supplemented with 10 mM Hepes, 5% (v/v) heat-inactivated fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol, 100 U penicillin, and 100 μg/ml streptomycin at 37 °C in 5% CO_2 (Merglen et al., 2004). HepG2 cells (Sigma) were grown in high glucose DMEM (Dulbecco's modified Eagle's medium) containing 10% (v/v) heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U penicillin, and 100 μg/ml streptomycin at 37 °C in 5% CO_2. HEK293T cells (Sigma) were grown similarly to the HepG2 cells, except for the L-glutamine concentration (4 mM). Transient transfection was performed as reported (Iacobazzi et al., 2005) using 0.5 μg of pGL3 promoter-LUC vector containing the CIC gene FOXA site. The extent of transfection was normalized by β-galactosidase activity (Infantino et al., 2011b). In RNA interference experiments, the specific pre-designed small interfering RNA (siRNA) targeting human FOXA1 (s6689, Ambion), was transfected in HepG2 cells using siPORT™ NeoFX™ Transfection Agent (Ambion). A siRNA (Cat. no. C6A-0126, Ambion) with no significant similarity to human, mouse, or rat gene sequences was used as negative control. To generate lentivirus, HEK293T cells were transfected with 0.5 μg of pLKO.1-FOXA1 (or control pLKO.1 vector) and 10 μl of MISSION Lentiviral Packaging Mix (Sigma) by using Fugene HD (Roche). Viruses were harvested 48 and 72 h after transfection. Viral titer was measured by the HIV p24 Antigen enzyme-linked immunosorbent assay (ELISA) (ZeptoMetrix). Transduction of INS-1 cells was performed with recombinant pLKO.1-FOXA1 lentivirus at a viral titer of approximately 10^5 transducing units/ml in the presence of 10 μg/ml polybrene/10 mM Hepes, pH 7.4. An empty lentivirus construct used at the same titer served as control (Iacobazzi et al., 2009a).