Long-Term Exposure to the Atypical Antipsychotic Olanzapine Differently Up-Regulates Extracellular Signal-Regulated Kinases 1 and 2 Phosphorylation in Subcellular Compartments of Rat Prefrontal Cortex

Fabio Fumagalli, Angelisa Frasca, Maria Spartà, Filippo Drago, Giorgio Racagni, and Marco Andrea Riva

Center of Neuropharmacology, Department of Pharmacological Sciences University of Milan, Milan, Italy (F.F., G.R., M.A.R.); Istituto di Ricovero e Cura a Carattere Scientifico, San Giovanni di Dio-Fatebenefratelli, Brescia, Italy (A.F., G.R.); and Department of Experimental and Clinical Pharmacology, School of Medicine, University of Catania, Catania, Italy (M.S., F.D.)

Received October 12, 2005; accepted January 3, 2006

ABSTRACT

Antipsychotics are the drugs of choice for the treatment of schizophrenia. Besides blocking monoamine receptors, these molecules affect intracellular signaling mechanisms, resulting in long-term synaptic alterations. Western blot analysis was used to investigate the effect of long-term administration (14 days) with the typical antipsychotic haloperidol and the atypical olanzapine on the expression and phosphorylation state of extracellular signal-related kinases (ERKs) 1 and 2 (ERK1/2), proteins involved in the regulation of multiple intracellular signaling cascades. A single injection of both drugs produced an overall decrease in ERK1/2 phosphorylation in different subcellular compartments. Conversely, long-term treatment with olanzapine, but not haloperidol, increased ERK1/2 phosphorylation in the prefrontal cortex in a compartment-specific and time-dependent fashion. In fact, ERK1/2 phosphorylation was elevated in the nuclear and cytosolic fractions 2 h after the last drug administration, whereas it was enhanced only in the membrane fraction when the animals were killed 24 h after the last injection. This effect might be the result of an activation of the mitogen-activated protein kinase pathway, because the phosphorylation of extracellular signal-regulated kinase kinase 1/2 was also increased by long-term olanzapine administration. Our data demonstrate that long-term exposure to olanzapine dynamically regulates ERK1/2 phosphorylation in different subcellular compartments, revealing a novel mechanism of action for this atypical agent and pointing to temporally separated locations of signaling events mediated by these kinases after long-term olanzapine administration.

Antipsychotic drugs represent the mainstay of pharmacotherapy for schizophrenia. Whereas first-generation (typical) drugs are mainly effective for positive symptoms, atypical agents also improve negative symptoms and cognitive deterioration (Keefe et al., 2006). Although these drugs act through the short-term blockade of several neurotransmitter receptors, functional recovering of schizophrenic patients develops over time, implying that neuroadaptive changes take place in selected brain regions (Meltzer, 1991). One possible target of the action of antipsychotic drugs might be represented by intracellular signaling pathways governing information shuffling from the synaptic cleft to the nucleus.

It has been shown that patients with schizophrenia have altered brain expression and/or phosphorylation of β-catenin and AKT-glycogen synthase kinase-3 (Cotter et al., 1998; Kozlovsky et al., 2000; Beasley et al., 2001; Emamian et al., 2004). The relevance of these pathways to schizophrenia is corroborated by the evidence that antipsychotics increase levels and/or the activation state of these proteins in the brain of experimental animals (Emamian et al., 2004; Kang et al., 2004; Alimohamad et al., 2005) regardless of the therapeutic class. In addition, antipsychotics regulate cAMP-dependent protein kinase-mediated signaling in different rat cerebral regions (Turalba et al., 2004).

Recent data have shown that the MAP kinase pathway might also be modulated by short-term administration of...
antipsychotics (Pozzi et al., 2003; Valjent et al., 2004; Browning et al., 2005). Mitogen-activated protein kinase and extracellular signal-regulated kinase (ERK) represent a critical crossroad of multiple signaling cascades involved in the regulation of different cellular processes spanning from cell proliferation, differentiation, and survival (Schaefler and Weber, 1999; Colucci-D’Amato et al., 2003; Sweatt, 2004) to synaptic plasticity and cognition (Valjent et al., 2001; Adams and Sweatt, 2002; Thomas and Huganir, 2004).

Because these proteins operate as multifunctional signaling integrators involved in the regulation of gene transcription (Sweatt, 2004), we investigated whether long-term administration of antipsychotics could alter their expression and phosphorylation state in the prefrontal cortex, a region that contributes most to the cognitive impairments observed in patients with schizophrenia (Weinberger et al., 2001).

Our results show that long-term treatment with the atypical antipsychotic olanzapine has specific modulatory effects on ERK1/2 phosphorylation that might account for the improvements in cognitive symptoms of schizophrenia produced by the drug.

Materials and Methods

Materials. General reagents were purchased from Sigma-Aldrich (Milano, Italy), and molecular biology reagents were obtained from Ambion (Austin, TX), New England Biolabs (Beverly, MA) and Promega (Milano, Italy). Olanzapine was obtained from Eli Lilly (Sesto Fiorentino, Italy); haloperidol was purchased from Sigma-Aldrich.

Animal Treatment and Drug Paradigms. Male Sprague-Dawley rats (Charles River, Calco, Italy) weighing 225 to 250 g were used throughout the experiments. Animals were housed for 2 weeks before any treatment and were maintained under a 12-h light/dark cycle with food and water available ad libitum.

For the short-term treatment, animals received a single injection only of either vehicle (saline), haloperidol (1 mg/kg), or olanzapine (2 mg/kg) and were killed by decapitation 30 min or 2 h later. For the long-term treatment, rats were injected daily with the drugs for 14 days and were killed 2 or 24 h after the last drug injection. Vehicle, haloperidol, and olanzapine were administered by subcutaneous injection. Although appropriate drug dosing in rats is controversial, in our experiments, drug doses were chosen in accordance with published protocols (Schotte et al., 1996; Bubser and Deutch, 2002; Kapur et al., 2003). All animal handling and experimental procedures were performed in accordance with the EC guidelines (EC Council Directive 86/609 1987) and with the Italian legislation on animal experimentation (Decreto Legislativo 116/92).

Preparation of Protein Extracts. Prefrontal cortex (approximately weight, 8 mg) was dissected from 2-mm slices (prefrontal cortex defined as Cg1, Cg3, and IL subregions, corresponding to the plates 6–9 of the atlas of Paxinos and Watson, 1996), immediately frozen on dry ice, and stored at −80°C. Different subcellular fractions were prepared as described previously (Maragnoli et al., 2004). Tissues were homogenized in a glass-glass potter in cold 0.32 M sucrose containing 1 mM HEPES solution, 0.1 mM EGTA, and 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4, in presence of a complete set of protease inhibitors and a phosphatase inhibitor cocktail. The homogenized tissue was centrifuged at 5000g for 10 min. The resulting pellet (P1), corresponding to the nuclear fraction, was resuspended in a buffer (20 mM HEPES, 0.1 mM dithiothreitol, and 0.1 mM EGTA) with protease and phosphatase inhibitors; the supernatant (S1) was centrifuged at 9000g for 15 min to obtain a clarified fraction of cytosolic proteins (S2), and the pellet, corresponding to the membrane fraction (P2), was resuspended in the same buffer used for P1. Total protein content was measured in the P1, S2, and P2 fractions with the Bio-Rad Protein Assay (Bio-Rad, Milano, Italy).

Western Blot Analysis. ERK1/2 protein analysis was performed on P1, S2, and P2 fractions as described previously (Pumagalli et al., 2005). Total protein concentrations were adjusted to the same amount for all samples (10 μg per lane). All of the samples were run on an SDS-8% polyacrylamide gel under reducing conditions, and proteins were then electrophoretically transferred onto nitrocellulose membranes (Bio-Rad). Blots were blocked with 10% nonfat dry milk and then incubated with primary antibody. The blots were first probed with antibodies against the phosphorylated forms of the protein and then stripped and reprobed with antibodies against total proteins of same type. ERK1 and ERK2 native forms were detected by evaluating the band density at 44 and 42 kDa, respectively, after probing with a polyclonal antibody (1:10,000, 2 h, room temperature) (Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were incubated for 1 h at room temperature with a 1:10,000 dilution of peroxidase-conjugated anti-rabbit IgG (Sigma-Aldrich). ERK1 and ERK2 phosphorylated forms were detected by evaluating the band density at 44 and 42 kDa, respectively, after probing with a monoclonal antibody (1:10,000, 4°C, overnight) (Santa Cruz Biotechnology). Membranes were incubated for 1 h at room temperature with a 1:10,000 dilution of peroxidase-conjugated anti-mouse IgG (Sigma-Aldrich).

MEK1/2 native form was detected by evaluating the band density at 45 kDa after probing with a polyclonal antibody (1:5000, 2 h, room temperature) (Cell Signaling Technology). Membranes were incubated for 1 h at room temperature with a 1:5000 dilution of peroxidase-conjugated anti-rabbit IgG (Cell Signaling Technology). MEK1/2 phosphorylated form was detected by evaluating the band density at 45 kDa after probing with a polyclonal antibody (1:1000, 4°C, overnight) (Cell Signaling Technology). Membranes were incubated for 1 h at room temperature with a 1:1000 dilution of peroxidase-conjugated anti-rabbit IgG (Cell Signaling Technology).

ERK1 and ERK2 phosphorylated immunocomplexes were visualized by chemiluminescence using the SuperSignal West Femto (Pierce Chemical, Rockford, IL), whereas MEK1/2 phosphorylated immunocomplexes were detected using the ECL Western Blotting kit (Amersham Life Science, Milano, Italy). ERK1, ERK2, and MEK1/2 native immunocomplexes were visualized by chemiluminescence using the ECL Western Blotting kit (Amersham Life Science) according to the manufacturer’s instructions.

Results were standardized to a β-actin control protein, which was detected by evaluating the band density at 43 kDa after probing with a polyclonal antibody with a 1:10,000 dilution (Sigma-Aldrich). Membranes were incubated for 1 h at room temperature with a 1:10,000 dilution of peroxidase-conjugated anti-mouse IgG (Sigma-Aldrich).

Statistical Analysis. Expression and phosphorylation state of ERK1/2 were measured using the Quantity One software from Bio-Rad. The mean value of the control group within a single experiment was set at 100, and the data of animals injected with olanzapine or haloperidol were expressed as “percentages” of saline-treated animals. The phosphorylation of ERK1/2 was expressed as a ratio between phosphorylated ERKs and total ERKs (pERKs/ERKs).

The total levels of ERKs were normalized with β-actin (ERKs/β-actin). The same analysis was carried out for the expression and phosphorylation state of MEK1/2.

Statistical evaluation of the changes produced by antipsychotic treatment on the phosphorylation state or expression of targets proteins was performed using a one-way analysis of variance (ANOVA) followed by Scheffe’s F test. Significance for all tests was assumed at p < 0.05.

Results

The major aim of the present study was to examine the subcellular expression and phosphorylation state of ERK1/2 in rat prefrontal cortex after treatment with the typical antipsychotic haloperidol and the atypical olanzapine. The purity of cellular compartment preparation was demonstrated
previously (Fumagalli et al., 2005). Both ERK isoforms were revealed by Western blot analysis, with a more intense immunoreactivity for ERK2.

We first analyzed the short-term modulation of ERK expression and phosphorylation by both antipsychotics, killing the animals 30 min and 2 h after drug treatment. Overall, short-term treatment with antipsychotics showed a generalized decrease of ERK1/2 phosphorylation, although subtle differences can be detected. In the nuclear fraction, haloperidol and olanzapine significantly reduced ERK1/2 phosphorylation 30 min (haloperidol: ERK1 = −30%, p < 0.05 and ERK2 = −29%, p < 0.05; olanzapine: ERK1 = −25%, p < 0.05, ERK2 = −25%, p < 0.05) and 2 h (haloperidol: ERK1 = −44%, p < 0.05 and ERK2 = −34%, p < 0.05; olanzapine: ERK1 = −51%, p < 0.05, ERK2 = −35%, p < 0.05) after injection (Fig. 1a and c). In the cytosolic compartment, the two antipsychotics did not produce any change in ERK1/2 phosphorylation 30 min after injection, whereas both drugs significantly decreased ERK2 phosphorylation (haloperidol = −32%, p < 0.05; olanzapine = −33%, p < 0.05), with a tendency toward a decrease for ERK1 2 h after drug treatment (Fig. 1c). A similar trend was also detected in the membrane fraction, at both of the time points investigated, although a statistically significant reduction was only found for ERK2 phosphorylation after olanzapine injection (−25%, p < 0.05) when examined 2 h after injection.

Although 30 min after administration of both drugs total levels of ERK1/2 were not changed (Fig. 1b), single injection of olanzapine increased total ERK1/2 levels in the nucleus, with a concomitant decrease of their levels in the cytosol 2 h later (Fig. 1d). Conversely, at this time point, the short-term administration of haloperidol did not produce any significant change of the levels for both ERK isoforms in any cellular compartments.

We then performed two different long-term treatments (14 days) with haloperidol and olanzapine that could be distinguished on the basis of the time of sacrifice from the last injection. We reasoned that these distinct experimental paradigms could allow us to dissect between mechanisms directly related to (2 h) and those independent from (24 h) the last drug administration.

Figure 2a shows a representative immunoblotting demonstrating the increased ERK1/2 phosphorylation 2 h after the last injection of a 2-week treatment with olanzapine in rat prefrontal cortex. Quantitative analysis demonstrated that long-term treatment with olanzapine significantly enhanced ERK1 (+59%, p < 0.01) and ERK2 (+35%, p < 0.01) phosphorylation in the nuclear fraction of this brain region (Fig. 2b), whereas in the cytosolic fraction, the increase was restricted to ERK2 (+25%, p < 0.05) (Fig. 2b). Conversely, long-term haloperidol treatment did not elicit any significant change of ERK1/2 phosphorylation in the nuclear or in the cytosolic fractions (Fig. 2b). Furthermore, at this time point, phosphorylation of ERK1/2 isoforms was not changed after long-term haloperidol or olanzapine in the membrane fraction (Fig. 2b). No changes were measured in the total levels of ERK isoforms with either drugs in the different subcellular fractions (Fig. 2c).

When the animals were killed 24 h after the last drug injection, olanzapine, but not haloperidol, up-regulated ERK1 and ERK2 phosphorylation (+58%, p < 0.05 and +68%, p < 0.05, respectively) only in the membrane fraction, whereas in the nuclear or cytosolic compartments, the levels of ERK1/2 phosphorylation decayed back to control level (Fig. 3b). No changes were measured in these experimental conditions in the total levels of ERK isoforms with either drugs in the different fractions examined (Fig. 3c).

To dissect out the molecular mechanisms underlying increased ERK1/2 phosphorylation produced by long-term olanzapine treatment, we analyzed the expression and phosphorylation state of MEK1/2, a kinase upstream of ERK1/2. The analysis of MEK1/2 phosphorylation in the cytosol, the subcellular compartment in which MEK1/2 is primarily located and enriched, revealed that olanzapine significantly
up-regulated MEK1/2 phosphorylation 24 h after the last drug treatment, whereas only a trend was observed 2 h after the last drug injection. No significant changes were produced by long-term olanzapine treatment on MEK1/2 total expression (Fig. 4c).

**Discussion**

Our results demonstrate that, in rat prefrontal cortex, ERK1/2 phosphorylation is selectively increased by long-term treatment with olanzapine, but not haloperidol, according to a finely tuned, compartment- and temporal-specific profile. The subcellular localization of such an effect is strictly correlated to the time of sacrifice from the last injection; in fact, such enhancement is specifically confined to the nuclear and cytosolic fraction 2 h after the last injection, but it is restricted to the membrane fraction when the animals are killed 24 h later.

A single injection of haloperidol or olanzapine produced an
overall reduction of ERK1/2 phosphorylation in the nuclear and cytosolic compartments, an effect that might be the consequence of the blockade of dopaminergic and serotonergic receptors. In the case of olanzapine, however, whereas the antipsychotic directly reduces ERK1/2 phosphorylation 30 min after drug injection, the effect observed 2 h later may be the result of increased protein expression. Based on these data, we suggest that olanzapine might promote protein translocation from the cytosol to the nucleus 2 h after treatment, presumably in an attempt to counteract the decreased ERK1/2 phosphorylation.

The different effect of single versus repeated injections of olanzapine is suggestive of the possibility that long-term treatment with the atypical antipsychotic might determine adaptive mechanisms that lead to the up-regulation of ERK1/2 phosphorylation in selected cell compartments.

The specific, compartmentalized increase in ERK1/2 phosphorylation poses an interesting question regarding the role of ERKs in the different subcellular fractions with respect to the mechanism of action of antipsychotic drugs. Evidence exists that activated ERK1/2 play distinct roles in the nucleus [in which they activate transcription factors or immediate early genes such as Elk-1 or c-fos (Sgambato et al., 1998a,b)], in the cytoplasm [in which they seem to be implicated in the regulation of cytoplasmic proteins involved in synaptic rearrangements such as MAP2 (Bhat et al., 1998) or Cdk5 (Veeranna et al., 2000)], or in the membrane fraction [in which, postsynaptically, they participate in the regulation of plasticity and learning (Komiyama et al., 2002) while presynaptically are involved in regulating neurotransmitter release (Schenk et al., 2005)]. To this regard, Harding and associates (2005) recently demonstrated that a different threshold of activation exists for ERK1/2 in different subcellular compartments that might explain the temporal redistribution of the activated kinase after long-term olanzapine administration.

Olanzapine, similarly to other novel antipsychotics, has a complex pharmacodynamic profile, thus posing an important question as to how it enhances ERK1/2 phosphorylation in prefrontal cortex. Direct blockade of dopamine D2 receptors is not likely to contribute to ERK1/2 changes, because haloperidol, a preferential antagonist of these receptors, is devoid of any effect. Although we cannot exclude a role for a specific receptor subtype, it could be hypothesized that the activation of ERKs in prefrontal cortex might represent the consequence of complex events involving direct receptor antagonism and enhancement of neurotransmitter release produced by olanzapine. In fact, it has been shown that olanzapine increases dopamine and norepinephrine release in rat prefrontal cortex (Bymaster et al., 1999), an effect that may contribute to the increase in ERK1/2 phosphorylation (Zhong and Minneman, 1999; Runyan and Dash, 2004). In addition, atypical drugs enhance glutamate transmission, thus facilitating N-methyl-D-aspartate responses in pyramidal cells of medial prefrontal cortex (Heresco-Levy, 2003; Ninan et al., 2003).

Increased ERK1/2 phosphorylation may be the result of a direct activation of the MAP kinase pathway, or it might depend on indirect modulation by other pathways involved in the phosphorylation of these proteins. Based on our data, we propose that increased phosphorylation of MEK1/2, the kinase upstream of ERK1/2, is responsible of the up-regulation of ERK1/2 phosphorylation. However, we cannot rule out the possibility that other mechanisms participate in such enhancement. Among the intracellular cascades relevant for the action of olanzapine, the pathway of cAMP-dependent protein kinase can increase ERK1/2 phosphorylation (Yao et al., 1998; York et al., 1998; Grewal et al., 1999; Robertson et al., 1999). Alternatively, enhanced phosphorylation of ERK1/2 after long-term olanzapine treatment might also result from the inhibition of protein phosphatases. Indeed,
long-term administration of atypical antipsychotics downregulates the expression of serine-threonine phosphatases, the enzymatic system responsible for ERK1/2 dephosphorylation (MacDonald et al., 2005).

Postmortem studies in patients with schizophrenia (Kyosseva et al., 1999) and preclinical investigations in animal models of the disease using glutamate N-methyl-D-aspartate receptor antagonists (Kyosseva et al., 2001; Ahn et al., 2005) have shown dysregulation of the ERK pathway, suggesting that it may represent a target for therapeutic interventions. In support of these molecular observations, clinical studies have demonstrated that patients with schizophrenia display abnormalities in prefrontal information processing (Weinberger et al., 2001), which could contribute to the cognitive impairments associated with the disease. Clinical data indicate that olanzapine and in general second-generation antipsychotics successfully improve specific cognitive domains in patients with schizophrenia, whereas haloperidol and classical neuroleptics are not effective (Purdon et al., 2000; Woodward et al., 2005). Given that the ERK pathway modulates cognition in the prefrontal cortex (Runyan and Dash, 2004), our data raise the possibility that olanzapine might improve cognitive processes via the up-regulation of ERK1/2 phosphorylation in the prefrontal cortex.

To sum up, we indicate ERK as a dynamic target of antipsychotic administration, unraveling a previously unappreciated degree of subcellular regulation promoted by olanzapine. Although the findings need to be confirmed and extended to other antipsychotic drugs, these results could explain, at least in part, the ability of atypical antipsychotics in alleviating some of the schizophrenic negative symptoms and improving cognitive dysfunctions.

The potential implication of increased ERK1/2 phosphorylation after long-term antipsychotic administration might extend from schizophrenia to other psychiatric conditions. To this end olanzapine, but not haloperidol, delays or prevents relapse during long-term maintenance therapy of bipolar disorder (McCormack and Wiseman, 2004; Bowden, 2005). It should be emphasized that histone and valproate, which are widely used for the treatment of bipolar disorder, increase ERK1/2 phosphorylation in rat prefrontal cortex after long-term administration (Einat et al., 2003), pointing to enhanced function of ERK pathway in this brain structure as a common event of the long-term therapeutic action of psychotropic drugs.

Acknowledgments

We thank Eli-Lilly (Italy) for the generous gift of olanzapine.

References


**Address correspondence to:** Dr. M. A. Riva, Center of Neuropharmacology, Department of Pharmacological Sciences, Via Balzaretti 9, 20133 Milan, Italy. E-mail: m.riva@unimi.it