Haloperidol regulates the phosphorylation level of the MEK-ERK-p90RSK signal pathway via protein phosphatase 2A in the rat frontal cortex

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Abstract
Haloperidol, a classical antipsychotic drug, affects the extracellular signal-regulated kinase (ERK) pathway in the brain. However, findings are inconsistent and the mechanism by which haloperidol regulates ERK is poorly understood. Therefore, we examined the ERK pathway and the related protein phosphatase 2A (PP2A) in detail after haloperidol administration. Haloperidol (0.5 and 1 mg/kg) induced biphasic changes in the phosphorylation level of mitogen-activated protein kinase kinase (MEK), ERK, and p90 ribosomal S6 kinase (p90RSK) without changing Raf-1 phosphorylation. Fifteen minutes after haloperidol administration, MEK-ERK-p90RSK phosphorylation increased, whilst PP2A activity decreased. At 60 min, the reverse was observed and the binding of PP2A to MEK and ERK increased. Higher dosages of haloperidol (2 and 4 mg/kg), affected neither MEK-ERK-p90RSK phosphorylation nor PP2A activity. Accordingly, PP2A regulates acute dose- and time-dependent changes in MEK-ERK-p90RSK phosphorylation after haloperidol treatment. These findings suggest the involvement of a dephosphorylating mechanism in the acute action of haloperidol.

Key words: Antipsychotics, mitogen-activated protein kinase, protein serine/threonine phosphatase 2A.

Introduction
Haloperidol is a classical antipsychotic drug used to treat an array of psychotic disorders. Traditionally, the clinical potency of antipsychotics was thought to be related to its capacity to antagonize the dopamine D2 receptor (Creese et al., 1976). Whilst the inhibitory effect on the receptor is instantaneous, its antipsychotic effect requires chronic treatment-inducing long-term adaptive changes (Meltzer, 1991), which are mediated via intracellular signalling pathways. Acute regulation of signalling pathways by antipsychotics is an important first step in inducing a chronic response, which includes changes in gene expression (Li et al., 2007). Therefore, understanding the acute effect of antipsychotics on the intracellular signalling cascades is important.

The extracellular signal-regulated kinase (ERK) signal pathway is thought to be involved in the mechanism of antipsychotics and psychotomimetics (Ahn et al., 2006; Beaulieu et al., 2006; Pozzi et al., 2003; Valjent et al., 2004). ERK participates in a variety of neuronal functions, including the regulation of gene expression, protein synthesis, and receptor modulation, which contribute to synaptic plasticity and adaptive behaviours such as learning and memory (Sweatt, 2004).

The acute effect of haloperidol, a D2 receptor antagonist, on ERK activity has also been investigated. However, findings on the relationship between the D2 receptor and the ERK pathway are mixed. Stimulation of the D2 receptor inhibited ERK phosphorylation in pituitary and striatal cell cultures (Banihashemi and Albert, 2002; Liu et al., 2002; Van-Ham et al., 2007), and haloperidol acutely up-regulated ERK phosphorylation in the striatum and frontal cortex of rodents.
(Pozzi et al., 2003; Valjent et al., 2004) and in cell lines (Yang et al., 2004). In contrast, activation of the D₄ receptor was also reported to increase ERK phosphorylation in vitro and in vivo (Cai et al., 2000; Wang et al., 2005) with ERK phosphorylation accordingly found to be reduced in mouse and rat prefrontal cortex after a single treatment with haloperidol (Fumagalli et al., 2006; Pascoli et al., 2005). These contradictory findings could have resulted from different experimental conditions such as the kind or dose of receptor-modulating agents and the duration of treatments. The reports concerning the effect of haloperidol on the ERK pathway also examined a limited range of haloperidol doses and time-points. Moreover, the regulators of the ERK pathway in response to haloperidol treatment remain to be examined. Therefore, we investigated the ERK pathway in more detail and the molecules regulating ERK following haloperidol treatment.

The phosphorylation of ERK is regulated by interplay between kinases and phosphatases. Raf-1, recruited by GTP-bound Ras, is the best-known upstream kinase activating the mitogen-activated protein kinase kinase (MEK)-ERK pathway. Phosphorylation of its Ser338 residue activates Raf-1, whilst hyperphosphorylation of Ser259 interferes with its activity (Baccarini, 2005). In addition, several phosphatases have been reported to regulate ERK phosphorylation in the brain, including protein serine/threonine phosphatase PP2A, mitogen-activated protein kinase phosphatase (MKP) MKP-1/2 and MKP-3, and protein tyrosine phosphatases (PTP) haematopoietic PTP (HePTP), striatal enriched PTP (STEP), and PTP-SL (Murphy and Blenis, 2006). Of these, protein phosphatase 2A (PP2A) has been suggested to be one of the key regulators of MEK-ERK phosphorylation in the brain (Mao et al., 2005; Pei et al., 2003). PP2A negatively regulates ERK activity through the dephosphorylation of MEK or ERK, whilst PP2A can also positively regulate ERK activity via dephosphorylation of inhibitory upstream kinases of ERK, such as Ser259-Raf-1 (Adams et al., 2005; Alessi et al., 1995; Sontag, 2001). Therefore, the effect of PP2A on ERK activity is determined by the interacting partner within the Ras-Raf-MEK-ERK signalling pathway.

We hypothesized that the changes in ERK phosphorylation could be time- and dose-dependent and dephosphorylating action could be involved in regulating ERK phosphorylation in response to haloperidol treatment. Based on this hypothesis, we examined the time- and dose-dependent changes in ERK phosphorylation in detail, as well as those of molecules upstream (Raf-1 and MEK) and downstream [p90 ribosomal S6 kinase (p90RSK)] from ERK in the rat frontal cortex, a suggested site for the common action of diverse antipsychotics (MacDonald et al., 2005). In addition, to examine the involvement of phosphatase in ERK regulation after haloperidol treatment, PP2A activity and the binding of PP2A to Raf-1, MEK, or ERK were examined.

Method

Animals and drug treatment

Male Sprague–Dawley rats (150–200 g) were grouped and maintained on a 12-h light–dark cycle with food and water freely available. The animals were treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Haloperidol (Sigma-Aldrich, Steinheim, Germany) 0.5, 1, 2, and 4 mg/kg dissolved with 0.3% tartaric acid and pH adjusted to 6.0 was injected into the rats intraperitoneally, whilst control animals received an equivalent volume of 0.3% tartaric acid at the same pH.

To examine the temporal pattern of changes, we observed the phosphorylation level 15, 30, 60, and 120 min after haloperidol treatment. Four animals were assigned to each treatment group (vehicle, 0.5, 1, 2, or 4 mg/kg haloperidol for each time-point) for immunoblot analysis. For selected treatment groups based on the immunoblot results, 3–5 different rats were used for PP2A activity assay, immunoprecipitation, and Raf-1 kinase activity assay.

Immunoblot analysis

Whole extracts of frontal cortex were used for immunoblot analysis. Frontal cortices were immediately homogenized in a glass–Teflon homogenizer in 10% v/w ice-cold RIPA (+) buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton, 1% sodium deoxycholate, and 0.1% SDS] containing 1 mM DTT, protease inhibitor cocktail (Sigma–Aldrich), and 1 mM PMSF (Sigma–Aldrich). Subsequent steps for immunoblot analysis was performed as described previously (Ahn et al., 2006). Antibodies against actin (Sigma–Aldrich), Raf-1, MEK1/2, ERK1/2, p90RSK, PP2A catalytic subunit (Santa Cruz Biotechnology, Santa Cruz, CA, USA), phospho-Raf-1 (Ser259 or Ser338), phospho-MEK1/2 (Ser217/221), phospho-ERK1/2 (Thr202/Tyr204), or phospho-p90RSK (Thr360/Ser364) (Cell Signaling Technology, Beverly, MA, USA) were used as primary antibodies at dilutions of 1:1000 to 3000. They were incubated overnight at 4 °C, and this was followed by a second incubation with anti-rabbit IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology).
The signal was detected with the ECL system (Pierce, Rockford, IL, USA).

**Immunoprecipitation**

Tissues were homogenized in RIPA buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 1 mM EGTA, 1 mM EDTA, and protease inhibitor cocktail (Sigma-Aldrich)], and 1 mM PMSF (Sigma-Aldrich). After pelleting the insoluble proteins at 20 000 g for 20 min, the supernatant was pre-cleared with protein A-agarose. The pre-cleared samples were immunoprecipitated overnight with antibodies against Raf-1, MEK1/2 or ERK1/2 antibody. The immunocomplexes were recovered using protein A- or G-agarose and analysed by immunoblotting using antibodies against PP2A, Raf-1, MEK1/2, or ERK1/2. The ratios of immunoreactivity of PP2A to that of Raf-1, MEK, or ERK in the immunoprecipitated samples were compared as described in the Statistical analysis section.

**Serine and threonine phosphatase assay**

PP2A activity was determined using a molybdate dye-based phosphatase assay kit (cat. no. V2460, Promega, Madison, WI, USA). We described the detailed procedure in a previous report (Kang et al., 2005). In addition, the specificity of the PP2A activity was evaluated via collateral assays using okadaic acid (OA; 1 and 5 μM) as a PP2A inhibitor. OA inhibits PP1 as well as PP2A (Suganuma et al., 1992). However, since the substrate in this kit, RRA(pT)VA, is a poor substrate for PP1, it can be presumed that the OA-sensitive measured activity originated from PP2A. The optical densities (ODs) of the samples were assessed using a plate reader fitted with a 630-nm filter.

**Raf kinase activity assay**

Raf-1 kinase activity was determined using a Raf-1 kinase assay kit (cat. no. 17-360, Upstate Biotechnology, Lake Placid, NY, USA) according to the manufacturer’s instructions. Briefly, tissue extracts were incubated with a Mg/ATP cocktail, inactive MEK1/2 substrate, and assay dilution buffer I for 30 min at 30 °C and then boiled with Laemmli’s sample buffer. Phospho-MEK1/2 in the reaction was detected by immunoblot analysis with anti-phospho-MEK1/2 antibody.

**Statistical analysis**

The results are expressed as relative ODs, which are the percentages of the ODs compared to vehicle values, and are reported as the mean ± S.E. The mean relative OD from the immunoblot analysis or phosphatase assay and the ratios of immunoreactivity from the immunoprecipitation analysis were compared using one-way analysis of variance (ANOVA) or two-way ANOVA considering the effect of dosage and time, and pairwise comparisons were performed using Tukey’s post-hoc test. p values < 0.05 were considered statistically significant. All tests were performed using SPSS 12.0 for Windows (SPSS, Chicago, IL, USA).

**Results**

Haloperidol affected the phosphorylation state of MEK1/2, ERK1/2, and p90RSK in the rat frontal cortex in a dose- and time-dependent manner. Haloperidol (0.5 and 1 mg/kg) transiently increased the immunoreactivities of p-Ser217/221-MEK1/2, p-Thr202/Tyr204-ERK1/2, and p-Thr360/Ser364-p90RSK at 15 min (all p < 0.05), and then decreased all of them below the vehicle control value from 30 min to 120 min after the treatment (all p < 0.05). Higher doses (2 and 4 mg/kg) did not induce significant changes in the phosphorylation level of any molecule examined at any time. No changes occurred in the immunoreactivities of total MEK1/2, ERK1/2, and p90RSK at any dosage until 120 min. The immunoreactivities of p-Ser338-Raf-1 and p-Ser259-Raf-1, a canonical upstream kinase of MEK, did not change significantly under any condition examined. The immunoreactivity of total Raf-1 also did not change (Figure 1).

A transient increase in phosphorylation followed by a long-lasting decrease suggested activation of an active dephosphorylating mechanism. Therefore, we examined the activity of PP2A after haloperidol treatment in the rat frontal cortex. The activity was examined 15 min and 60 min after 0.5 or 2 mg/kg haloperidol treatment. The doses and times were selected based on the phosphorylation data reported above. At the lower dose (0.5 mg/kg), PP2A activity was reduced significantly compared to the vehicle control (p = 0.02) at 15 min, whilst it increased significantly at 60 min (p < 0.01). A higher dose (2 mg/kg) of haloperidol did not elicit any changes in the activity of PP2A. Collateral assays performed in the presence of OA, a PP2A inhibitor, resulted in the total abolition of phosphatase activity. Although OA also inhibits PP1, the substrate used for phosphatase assay, RRA(pT)VA, is a poor substrate for PP1. Thus, it was confirmed that the phosphatase activity emanated from the OA-sensitive PP2A (Figure 2).

To demonstrate the involvement of PP2A in the MEK-ERK pathway after haloperidol treatment more
directly, we next investigated whether these molecules are co-precipitated, and if so, whether haloperidol treatment affects the binding. The dephosphorylating activity of PP2A requires a physical interaction with the substrate molecule (Sontag, 2001). Protein samples collected 60 min after 0.5 or 2 mg/kg haloperidol were immunoprecipitated with antibodies against Raf-1, MEK1/2, or ERK1/2, and then immunoblotted with antibodies against PP2A, Raf-1, MEK1/2, or ERK1/2. The binding of PP2A to MEK1/2 increased under the low-dose condition (0.5 mg/kg) compared to the vehicle condition ($p<0.01$), whilst it did not
change under the high-dose condition (2 mg/kg). The binding of PP2A to ERK1/2 showed the same pattern. However, the binding of PP2A to Raf-1 did not change significantly under either low-dose (0.5 mg/kg) and high-dose (2 mg/kg) conditions. The immunoreactivity of PP2A in the original lysate used for the immunoprecipitation analysis did not change (Fig. 3).

In addition, Raf-1 kinase activity was examined to determine whether the changes in the phosphorylation of MEK-ERK-p90RSK and in the activity of PP2A are related to Raf-1 activity. As expected by the finding of no changes in immunoreactivities of p-Ser338-Raf-1 and p-Ser259-Raf-1, Raf-1 kinase activity did not alter significantly at 15 min and 60 min after haloperidol (0.5 and 2 mg/kg) treatment (Fig. 4).

Discussion

Haloperidol (0.5 and 1 mg/kg), induced biphasic changes in the phosphorylation level of MEK-ERK-p90RSK and in the activity of PP2A. At an earlier time (15 min), the phosphorylation of the kinases was elevated, with decreased activity of PP2A. At a later time (60 min), the reverse was observed. At this time, the binding of PP2A to its potential substrates, MEK or ERK, also increased. However, higher dosages of haloperidol (2 and 4 mg/kg) affected neither the phosphorylation status of MEK-ERK-p90RSK nor the activity of PP2A. Our findings indicate the involvement of PP2A in the dose- and time-dependent changes of MEK-ERK-p90RSK phosphorylation in response to haloperidol treatment.

Our results concerning the dose- and time-dependent effect of haloperidol on the ERK pathway encompass the previous inconsistent findings. At a dosage of 0.5 or 1 mg/kg, haloperidol either increased ERK phosphorylation at 15 min (Valjent et al., 2004) or decreased ERK phosphorylation in the rat or mouse frontal cortex at 30 min or 120 min (Fumagalli et al., 2006; Pascoli et al., 2005). These results seemed to contradict each other. In our experiment, however, we found that these are not contradictory but instead sequential effects: an early (15 min) increase and late (30 min and thereafter) decrease. It can also be postulated that the dose itself can affect the time-course of the phosphorylation response, since a
The phosphorylation profiles of its possible substrates, MEK and ERK, suggested that this change in PP2A activity could be a regulator of the ERK signalling system after haloperidol treatment. The level of MEK-ERK-p90RSK phosphorylation showed the inverse pattern to PP2A activity level. The increased PP2A activity observed at a later time when MEK/ERK phosphorylation decreased was prominent, and, moreover, was accompanied by an increased physical association between PP2A and MEK-ERK. However, the decreased PP2A activity observed at an earlier time when ERK phosphorylation increased was not prominent, suggesting the possible involvement of another mechanism, in addition to regulation via PP2A, in ERK activation. In addition, the binding of PP2A to Raf-1 did not change significantly after haloperidol treatment. Taken together with the finding of no changes in Raf-1 activity, the regulatory action of PP2A on the MEK-ERK pathway in response to haloperidol seems to be independent of Raf.

The time-dependent changes can be related to the action of haloperidol on dopamine receptors. As haloperidol initially antagonized the presynaptic inhibitory D2 autoreceptor, haloperidol treatment induced a rapid and transient increase in dopamine transmission in the rat brain (Garris et al., 2003; Lidsky and Banerjee, 1993). Under the blockade of the D2 receptor by haloperidol, increased dopamine might stimulate the D1 receptor, which is responsible for the ERK activation that occurs in response to dopaminergic stimulation (Valjent et al., 2000, 2005; Zhang et al., 2004). However, this initial response to autoreceptor blockade undergoes rapid tolerance, and a progressive decrease in dopamine level ensues (Garris et al., 2003; Imperato and Di Chiara, 1985). This process may be associated with the biphasic effect of haloperidol (0.5 and 1 mg/kg) on ERK and PP2A. In addition, the initial increase of ERK phosphorylation may also be a consequence of the inhibition of the acute D3 receptor-Gi protein-mediated inactivation of ERK (Banihashemi and Albert, 2002; Van-Ham et al., 2007). Taken together, the sequential alterations in dopaminergic transmission and the combined involvement of the D1 and D2 receptors following haloperidol treatment may be related to the biphasic changes in ERK phosphorylation.

The pharmacological action of haloperidol is believed to be primarily mediated by D2 receptor antagonism. However, haloperidol also affects α1-adrenergic, D4, and 5-HT2 serotonin receptors, although to a lesser extent (Horacek, 2006; Ohta, 1976; Reimold et al., 2007; Schotte et al., 1993). Stimulation of the D1 or α1-adrenergic receptor activated ERK (Valjent et al., 2000, 2005; Zhang et al., 2004; Zhong et al., 2004).
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and Minneman, 1999), and haloperidol blocked D1 receptor-Gs, protein-related signalling (Cai et al., 1999; Cussac et al., 2004) and affected α1-adrenoceptor-coupled signals in a dose-dependent manner (Borda et al., 1999; Nalepa, 1993). In addition, serotonergic modulation also affected ERK activity (Beaulieu et al., 2006; Johnson-Farley et al., 2005; Quinn et al., 2002). The receptor occupancy profile of haloperidol is dose-dependent (Schotte et al., 1993), and this complex effect on neurotransmitter receptors may contribute to the dose-dependent effect of haloperidol on intracellular signalling alterations, a process that needs further clarification.

PP2A has been reported to play an important role in dopamine receptor signalling. PP2A regulates D4 class-receptor-mediated Akt phosphorylation through the formation of a signalling complex with β-arrestin (Beaulieu et al., 2005). β-arrestin also acts as a scaffold linking the G-protein coupled receptors (GPCRs), such as β-adrenergic or angiotensin receptors, and the ERK pathway (Ahn et al., 2004; Shenoy et al., 2006), and thus the possible linking between the dopamine receptor, another GPCR, and ERK through β-arrestin was suggested (Bibb, 2005). Therefore, it seems valuable to study whether β-arrestin is involved in the effect of haloperidol on the ERK pathway via PP2A. In addition, PP2A is also involved in the phosphorylation-mediated regulation of the dopamine transporter, which modulates the dopamine neurotransmission system (Vaughan, 2004). Accordingly, PP2A can regulate dopamine receptor signalling. Despite the important roles of PP2A in the dopamine neurotransmission system, the effect of antipsychotics on PP2A remains to be clarified.

Previously, we reported the acute regulation of PP2A activity after electroconvulsive seizure, another psychotropic treatment modality (Kang et al., 2005). Together with our present finding, PP2A might be a valuable target for clarifying the acute intracellular action of antipsychotic treatment. There are only a few reports concerning the chronic effect of antipsychotics on PP2A. The down-regulation of protein serine/threonine phosphatase in the rat frontal cortex (MacDonald et al., 2005) or reductions in calcineurin A expression without changes in PP2A expression in the rat striatum and prefrontal cortex (Rushlow et al., 2005) after chronic antipsychotic treatment, including haloperidol, has been reported. However, acute regulation differs from the chronic response, and further investigations are necessary to understand the role of PP2A in the action mechanism of antipsychotics.

Taken together, PP2A might be a key regulator of the MEK-ERK-p90RSK pathway in response to haloperidol treatment, but further studies are required to clarify the detailed molecular mechanism by which haloperidol regulates PP2A activity. Our finding concerning the acute effect of haloperidol on PP2A should increase understanding of the possible involvement of a dephosphorylating mechanism in the acute action of antipsychotics.

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Statement of Interest

None.

References


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