Chronic Treatment with Fluvoxamine Stimulates Phosphorylation of Ser\textsuperscript{473} and Thr\textsuperscript{308} of Akt in the Rat Cerebral Cortex

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Abstract

The clinical therapeutic effect of most antidepressant drugs only develops after several weeks of administration, but little is known about the intracellular signal transduction in neuron. Here, we examined the effect of chronic treatment with fluvoxamine (FLV), a selective serotonin reuptake inhibitor (SSRI) used to treat depression, on the serine-threonine kinase Akt (also known as protein kinase B), which plays key roles in various cellular processes including neurotransmitter release, apoptosis and transcription. Male Wistar rats received injections of FLV once daily for 3 weeks, and the protein and phosphorylation levels of Akt in the temporal cortex were determined by immunoblot analysis. The protein expression level of Akt was unchanged from the control after FLV treatment, but phosphorylation of Ser\textsuperscript{473} and Thr\textsuperscript{308} of Akt was increased compared with the control. The numbers of neurons immunostained for Akt in the somatosensory cortex (layer II/III) were similar in the control and FLV groups, but the numbers of phospho Akt-Ser\textsuperscript{473} and phospho Akt-Thr\textsuperscript{308} immunoreactive neurons were increased in the FLV group compared with the control. The increase of phospho Akt was localized in the nucleus. The level of brain-derived neurotrophic factor (BDNF), an upstream mediator of Akt activation, was also significantly increased in the FLV group. Our results indicate that chronic FLV treatment activates Akt by inducing phosphorylation of Ser\textsuperscript{473} and Thr\textsuperscript{308}, and the activated Akt is translocated to the nucleus of neurons in the temporal cortex, this may lead to changes of transcriptional regulation.

Keywords

Akt, fluvoxamine, selective serotonin reuptake inhibitor, phosphatidylinositol 3-kinase, brain-derived neurotrophic factor

Introduction

Akt, also known as protein kinase B (PKB), is an oncogenic serine-threonine kinase originally cloned from the retrovirus AKT8. It is a direct target of phosphatidylinositol 3-kinase (PI3K) and is activated in response to a variety of growth factors, including insulin and brain-derived neurotrophic factor (BDNF). BDNF activates PI3K via the receptor tyrosine kinase TrkB\textsuperscript{1,2}. Akt is translocated from cytosol to the plasma membrane, mediated by the binding of PI3K products, namely
phosphatidylinositol (3, 4) bisphosphate [PtdIns (3, 4)P₂] and phosphatidylinositol (3, 4, 5) trisphosphate [PtdIns (3, 4, 5)P₃], to the PH domain of Akt. The binding also leads to a conformational change that exposes Akt-Ser⁴⁷³ and Akt-Thr³⁰⁸ to the membrane-associated and constitutively activated upstream kinase phosphoinositide-dependent kinase-1 (PDK1) and PDK2, respectively. The phosphorylated Akt detaches from the membrane, and is translocated to the nucleus³⁴⁶. Key roles for Akt in many cellular processes, including neurotransmitter release, apoptosis³⁵ and transcription are well established.

Recent studies suggested a role of BDNF in depression. The expressions of BDNF were lower in depressed patients, and antidepressant treatment increases serum BDNF concentration in these patients⁶. Chronic administration of selective serotonin reuptake inhibitors (SSRIs), leads to upregulation of BDNF mRNA³, and Yagasaki et al. reported that fluvoxamine (FLV), one of SSRI, enhanced the BDNF-induced glutamate release and increased intracellular concentration of Ca²⁺ in cultured cortical neurons⁷. In the present work, we investigated the effect of chronic treatment with FLV on the protein and phosphorylation levels of Akr, and the protein level of BDNF, in the rat cerebral cortex. We also immunohistochemically examined the translocation of phosphorylated Akt to the nucleus in neurons of layer II/III of the somatosensory cortex in the FLV-treated rats.

Material and methods

Animals

Male Wistar rats (150–300 g) received s.c. injections of FLV (5 mg/kg) in saline water once daily for 3 weeks, consultation of the method of Belowskii⁹. Control animals were injected saline water alone. Both groups were given food and water ad libitum.

The experiments were conducted in accordance with the guiding principles for the care and use of laboratory animals approved by St. Marianna University School of Medicine and the recommendations of the Declaration of Helsinki.

Preparation of cytosol and nuclear fractions of rat brain

Rats were sacrificed by decapitation under pentobarbital anesthesia, and the temporal cortex was immediately removed. According to the method of Manji⁹, the tissue was homogenized using a glass-Teflon homogenizer (12 strokes) in 20 mM Tris-HCl buffer (pH 7.5), 0.5 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 10% glycerol, 320 mM sucrose, 100 nM calyculin A, 10 μg/ml leupeptin, 5 mM phenylmethylsulfonyl fluoride (PMSF) and 100 μg/ml trypsin inhibitor. The homogenate was then centrifuged at 1,000 x g for 10 min. The supernatant was recentrifuged at 100,000 x g for 30 min. The resulting supernatant was subjected to DEAE Sepharose fast flow column (Pharmacia) chromatography with elution buffer containing 20 mM Tris-HCl buffer (pH 7.5), 0.5 mM EGTA, 0.5 mM EDTA, 1 mM DTT, 10% glycerol, 1 mM PMSF and 0.8 M KCl. The eluate was used as the cytosol fraction.

The method described by Dignam¹⁰ was used to prepare the nuclear fraction. Tissue was homogenized using a glass-Teflon homogenizer (12 strokes) in 10 mM HEPES buffer (pH 7.5), 1.5 mM MgCl₂, 10 mM KCl and 0.5 mM DTT. The homogenate was centrifuged at 1,000 x g for 25 min, and the pellet was resuspended in 20 mM HEPES buffer (pH 7.5), 25% glycerol, 420 mM KCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 5 mM PMSF, 10 μg/ml leupeptin, 100 μg/ml trypsin inhibitor, and 100 nM calyculin A. The suspension was stirred gently for 60 min and then centrifuged at 25,000 x g for 30 min, and the supernatant was used as the nuclear fraction.

Immunoblotting

The protein levels were determined by immunoblot analysis with antibodies against Akt1/2 (H-136) (Santa Cruz Biotechnology, Inc.), phospho-protein kinase B (pSer473) (Affinity BioReagents, Inc.), phospho-protein kinase B (pThr308) (Affinity BioReagents, Inc.) and BDNF (N-20) (Santa Cruz Biotechnology, Inc.). Each sample (20 μg) was resolved by SDS-PAGE and the separated proteins were transferred to nitrocellulose membranes. The membranes were blocked with 50 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl and 10% skim milk for 1 h, then incubated with each antibody at 1:500 - 1:1,000 dilution in for 6 h at 4°C in the 50 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl. Proteins were detected using the ECL system (Amersham Corp.) with horseradish peroxidase-conjugated secondary antibody (Amersham Corp.) at 1:2,000 dilution. Immunoblots were quantified by densitometric analy-
Immunohistochemistry
For immunocytochemical study, rat brain was perfused with 4% paraformaldehyde phosphate buffer solution under pentobarbital anesthesia. The brain was removed and frozen. The coronal sections of somatosensory cortex, a part of temporal cortex, were prepared with a microtome (Komatsumi Electronics, Japan) at a thickness of 40μm. All of the sections were washed three times in phosphate-buffered saline (PBS) for 5 min at room temperature and treated with ABC Reagent (Vectastain ABC kit, Vector Laboratories, Inc., Burlingame, CA) according to the manufacturer’s instructions. Nonspecific binding sites were blocked with goat serum albumin in PBS for 20 min. The sections were incubated overnight at 4°C with Akt1/2 (H-136), phospho-Akt (Ser473) (Cell Signaling TEC), and phospho-protein kinase B (p Thr 308) (Affinity BioReagents) antibodies diluted 1:100 in blocking solution as primary antibodies, and then washed three times in PBS for 5 min each at room temperature. The sections were incubated with biotinylated anti-rabbit secondary antibody diluted in 1% BSA in PBS for 30 min and washed three times in PBS for each 5 min at room temperature. They were then incubated with avidin-biotin-peroxidase complex solution for 30 min, washed three times in PBS for each 5 min at room temperature, and further incubated with the DAB reagent until staining was optimal as determined. The sections were mounted on glass slides, and the numbers of stained neurons in the somatosensory cortex (layer II/III) were counted in an arbitrary grid of 200μm × 200μm by light microscope.

Statistical analyses
Densitometric data and numbers of stained neurons were expressed as a percentage of the corresponding control value, which was taken to be 100%. Statistical analysis was performed with ANOVA (Fisher PLSD) using StatView. The level of statistical significance was defined as P<0.05. Data are presented as mean±S.E.M.

Results
Expression of BDNF, Akt and phosphorylation of Akt following chronic FLV treatment
BDNF in the cytosol fraction was identified by immunoblot analysis at a position corresponding to 14 kDa. The expression of the BDNF in the chronic FLV treatment group (3 weeks) was increased to 121±42.3% compared with the control (100±22.0%) (P=0.035) (n=6) (Fig. 1).

In the cytosol of the chronic FLV treatment group, the expression of Akt was also increased. The expression of phospho-Akt (Ser473) was also increased, indicating the phosphorylation of Akt. The expression of phospho-protein kinase B (p Thr 308) was also increased, indicating the phosphorylation of protein kinase B.
group, the density of the Akt band was expressed to 72±33.6% compared with the control (100±32.7%) (P=0.17) (n=7) (Fig. 2A). The levels of total Akt protein and β-Aktin protein were unaffected. However, the levels of phospho Akt-Ser473, Akt-Thr308 increased to 153% compared with the control (P=0.017) and 137.5% compared with the control (100±16.9%) (P=0.025) (n=7) (Fig. 2A).

The nuclear fraction also showed a change of Akt protein level in the chronic FLV treatment group at 106.5±9.0% compared with the control at 100±6.5% (P=0.65) (n=7) (Fig. 2B). Phospho Akt-Ser473 and phospho Akt-Thr308 were increased to 185.3±66.9% compared with the control (100±18.3%) (n=7) (P=0.013) and 193.7±40.6% compared with the control (100±37.0%) (n=7) (P=0.0077), respectively (Fig. 2B).

**Immunohistochemical evaluation of Akt phosphorylation following chronic FLV treatment**

The numbers of stained neurons in the somatosensory cortex were counted in an arbitrary grid of 200 µm x 200 µm at low-magnification images (×100) of sections (Fig. 3). Immunohistochemical staining for Akt revealed the number of stained neurons. Chronic FLV treatment group was at 96±3 compared with the control at 100±5 (P=0.52). The number of phospho Akt-Ser473 and Akt-Thr308-immunoreactive neurons was increased to 134±3 compared with the control at 100±6 (P=0.0003) (n=6) and 125±4% in compared with the control at 100±3 (P=0.0002), respectively.

High-magnification images (x400) revealed immunoreactivity for Akt in cytosol and nuclear fractions (Fig. 4). The levels of total Akt immunoreactivity in cytosol and nuclear fractions from the so-

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**Fig. 2.** Change in the Akt protein, Akt phosphorylated at Ser473 (p-Akt-Ser473) and Thr308 (p-Akt-Thr308) expressions in rat temporal cortex after FLV treatment. Immunoblots were quantified by densitometric analysis (control=100%). Values are means ± S.E.M. of seven rats. Immunoblot analysis was carried out with anti-Akt antibody at dilution of 1:500, anti-phospho Akt-Ser473 antibody and anti-phospho Akt-Thr308 antibody at a dilution of 1:1,000. Only the part of the gel containing each band is shown and each molecular weight is 60 kDa. The cytosol and nuclear fractions were subjected to SDS-PAGE. The levels of total Akt protein and β-Aktin protein were unaffected in the cytosol and nuclear fractions. However, the levels of phospho Akt-Ser473, Akt-Thr 308 increased compared with control in each fraction: *P<0.05.  **P<0.01**
matosensory cortex (layer II/III) showed similar values between FLV (Fig. 4B) and control groups (Fig. 4A). Phospho Akt-Ser473 and phospho Akt-Thr308 immunoreactivity was detected mainly in the cytosol fraction of the control group (Fig. 4C and E), and mainly in the nuclear fraction of the FLV group (Fig. 4D and F).

**Discussion**

BDNF is an upstream mediator of Akt activation, acting via TrkB and PI3K, and its level is known to be decreased in depressed patients, and increased by antidepressant treatment. 5-HT receptors in the parietal cortex and other neocortical areas play an important role in regulating the expression of BDNF mRNA. We found here that the protein level of BDNF was increased after treatment with FLV, compared with the control.

We found out that FLV induced Akt-Ser473, Akt-Thr308 phosphorylation after chronic treatment in the rat temporal cortex on the immunoblot. Immunohistochemical findings showed an increase of phosphorylated Akt in the nucleus after treatment with FLV.

Meier R et al reported that IGF-1 (insulin-like growth factors) stimulated PI-3 kinase through receptor tyrosine kinases. That IGF-1 phosphorylated Akt on Ser473 and Thr308 in REF52 cells. The phosphorylated Akt is translocated from cytosol to the nucleus after activation of Akt. Our data suggested that FLV induced the phosphorylation of Akt-Ser473, Akt-Thr308 and activated Akt is translocated from cytosol to the nucleus. Our results were similar phenomenon with the result which Meier R et al showed by the cultured cell. Therefore, it was considered that the same thing had occurred within a cultured cell and the rat brain.

Recent studies reported that Akt regulates the expression of various genes, chronic treatment with antidepressants are also thought to influence gene transcription. In conclusion, our findings here are consistent with the idea that chronic FLV treatment induces BDNF-mediated activation of Akt via phosphorylation of Ser473 and Thr308 of Akt, and the activated Akt is translocated to the nucleus of neurons in the somatosensory cortex, this in turn may lead to changes of transcriptional regulation.

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Fig. 4. Higher magnification images (×400) of immunoreactive neurons in rat somatosensory cortex (layer II/III). In Akt immunoreactivity, the cell is seen equally in a control rat (A) and an FLV-treated rat (B), respectively. In phospho Akt-Ser473 immunoreactivity, the cytosol is seen mainly in a control rat (C), but the nucleus is seen mainly in an FLV-treated rat (D). In phospho Akt-Thr308 immunoreactivity, the cytosol is seen mainly in a control rat (E), but the nucleus is seen mainly in an FLV-treated rat (F).


Fluvoxamine 慢性投与後ラット大脳皮質におけるAkt-Ser$^{473}$, Akt-Thr$^{308}$ のリン酸化の増加

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抄録
抗うつ薬は臨床的には、数週間以上経たないと効果がでないが、その細胞内情報伝達系機構については十分に解明されていない。そこで我々は、新規抗うつ薬である selective serotonin reuptake inhibitor (SSRI) の一種、fluvoxamine (FLV) 長期投与した際の Akt (protein kinase B) の変化について検討した。FLV を雄性ウイスターラットに3週間投与し、Akt とリン酸化Ser$^{473}$ Akt, Thr$^{308}$ Akt の変化をイムノプロット法と免疫染色法を用いて測定した。イムノプロット法では、リン酸化 Ser$^{473}$ Akt, Thr$^{308}$ Akt はラット側頭葉の細胞質では、各々 153 %, 138 %増加し、核成分では、各々 185 %, 193 %増加していた。免疫染色法では二次体性感覚皮質 (II/III 層) にて、各抗体で染色される細胞数は、リン酸化 Ser$^{473}$ Akt が 134 %、リン酸化 Thr$^{308}$ Akt が 125 %各々増加していた。FLV の3週間投与後、Akt の上流にある脳由来神経栄養因子 (BDNF) が 121 %増加していた。FLV 慢性投与後、ラット側頭葉において、Ser$^{473}$ Akt, Thr$^{308}$ Akt のリン酸化が増加し、核成分での Akt のリン酸化が増加していたので、FLV により Akt が活性化され、核に移行したと考察した。