Up-Regulation of Tryptophan Hydroxylase Expression and Serotonin Synthesis by Sertraline

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ABSTRACT

The neurotransmitter serotonin is involved in a variety of brain functions, and abnormal changes in serotonin neurotransmission are associated with an array of psychiatric disorders, including depression. Sertraline is a selective serotonin reuptake inhibitor (SSRI) and an effective antidepressant. Sertraline increases the serotonin concentration in the synaptic cleft by a short-term action; however, clinical improvement is observed only after several weeks, suggesting that the therapeutic effect may be caused by long-term alterations in serotonin transmission. We determined the effects of sertraline on serotonin synthesis in vivo and in vitro. Long-term treatment of rats with sertraline up-regulated mRNA and protein levels of the serotonin-synthesizing enzyme tryptophan hydroxylase (TPH), as determined by in situ hybridization and immunocytochemistry, respectively. In vitro studies using RBL-2H3 cells also showed an increase in mRNA and protein levels of TPH by sertraline, as determined by Northern blot and immunoblot analyses, respectively. This was accompanied by increases in the levels of TPH enzymatic activity and total serotonin. These data demonstrate that in addition to the known short-term action as an uptake blocker, sertraline also exerts a long-term effect on the serotonin neurotransmission by enhancing serotonin synthesis. A similar effect was observed with another SSRI, fluoxetine, but not with the non-SSRI chlorpromazine. The up-regulation of TPH gene expression by sertraline was attenuated by the protein kinase A (PKA) inhibitor N-[2-(p-bromocinnamylamine)-ethyl]-5-isoquinolinesulfonamine, suggesting that a mechanism involving the PKA signaling pathway might at least in part mediate the long-term therapeutic action.

The neurotransmitter serotonin is believed to be involved in a variety of brain functions, including the control of mood, aggression, anxiety, pain, cognition, appetite, thermoregulation, and sexual behavior. The role of changes in serotonin neurotransmission in psychiatric disorders such as depression, schizophrenia, eating disorders, panic disorder, and obsessive-compulsive disorder, as well as suicide and aggression, is well recognized (van Praag, 1994; Heninger, 1995). The symptoms of these disorders are effectively alleviated by the class of drugs known as selective serotonin reuptake inhibitors (SSRIs) (Masand and Gupta, 1999).

Sertraline [(1*S*-*cis*)-4-(3,4-dichlorophenyl)-1,2,3,4-tetrahydro-*N*-methyl-1-nanphthalenamine hydrochloride] is the most potent inhibitor of serotonin reuptake (Hyttel, 1994). This drug is highly effective in alleviating depression as well as anxiety, obsessive-compulsive disorder, and panic disorder, and it has good side effect and tolerability profiles.

The action of the SSRIs on the serotonin system has been presumed to occur via the inhibition of synaptic reuptake of serotonin, thereby increasing the synaptic serotonin concentration and consequently enhancing the efficacy of the serotonin transmission. However, whereas the reuptake inhibition should occur immediately, the clinical improvement is observed only after several (2–6) weeks (Fuller and Wong, 1987). Furthermore, in animals, prolonged administration of SSRIs leads to an increase in the synaptic serotonin level, but the drugs that are administered on a short-term basis do not (Briley and Moret, 1993). Because of this delayed therapeutic effect, it has been suggested that alterations in serotonin transmission might occur by a long-term mechanism, such as changes in expression of the involved gene(s).

To date, most studies of the long-term effects of SSRIs have focused on changes in serotonin transporter and receptors, which have often yielded conflicting results. Investigations of the effects of SSRIs on expression or ligand binding activity of the serotonin transporter have reported increases (Hrdina and Vu, 1993; Lopez et al., 1994), decreases (Lesch et al., 1993; Benmansour et al., 1999), or no change (Spurlock et al., 1994). Long-term antidepressant treatment is reported to down-regulate the presynaptic serotonin autoreceptors (Neumaier et al., 1996; Anthony et al., 2000) and to enhance the

ABBREVIATIONS: SSRI, selective serotonin reuptake inhibitor; TPH, tryptophan hydroxylase; PKA, protein kinase A; H-89, N-[2-(p-bromocinnamylamine)-ethyl]-5-isoquinolinesulfonamine; PBS, phosphate-buffered saline; SSC, standard saline citrate; MOPS, 3-(N-morpholino)propanesulfonic acid.

electrically evoked release of serotonin (Blier and Bouchard, 1994), whereas others have reported that the autoreceptors remain functional (Hjorth and Auerbach, 1999).

Another mechanism by which the SSRIs might influence serotonin transmission is via augmentation of serotonin synthesis, which would in turn cause increased availability of the neurotransmitter in the synaptic cleft. Serotonin is synthesized from the amino acid L-tryptophan by the rate-limiting enzyme L-tryptophan, tetrahydropteridine:oxygen oxidoreductase (5-hydroxylating) (EC 1.14.16.4) [tryptophan hydroxylase (TPH)] followed by aromatic L-amino acid decarboxylase. Gartside et al. (1992) demonstrated that changes in the level of TPH activity can lead to corresponding alterations in the amount of serotonin released into the synaptic space. Hence, TPH can influence the efficacy of the serotonin neurotransmission and, consequently, drugs that modify activity and/or gene expression of TPH can have long-term effects on the serotonin system. Previous studies have demonstrated that both TPH activity (Florez and Takahashi, 1996; Valenciano et al., 2000) and gene expression (Foguet et al., 1993; Florez and Takahashi, 1996; Green et al., 1996) are increased by the cAMP/protein kinase A (PKA) signal transduction system.

Only one study so far has reported on the effect of SSRIs on TPH gene expression (Spurlock et al., 1994). These investigators observed the absence of changes in the TPH mRNA level in animals treated with the SSRIs citalopram and fluvoxamine, as determined by Northern blot analysis by the use of RNA isolated from whole rat brain. Because the cell bodies of TPH-expressing neurons are localized to a very small region called the raphe nucleus, detection of changes in these neurons is often difficult because of the dilution of signals by the relatively overwhelming number of the nonserotonergic cells. Techniques that allow observation in situ, such as in situ hybridization and immunocytochemistry, would provide a more accurate assessment of any alterations. In addition, because data obtained from in vivo studies are often influenced by other factors such as stress and handling, corroboration with in vitro results would be beneficial.

Unfortunately, no cell line derived from the dorsal raphe with sufficiently detectable levels of serotonin, TPH, and the serotonin transporter is available thus far. On the other hand, RBL-2H3 cells are basophilic leukemia cells and have been extensively studied for their ability to produce serotonin. These cells have been shown to contain serotonin and TPH (Hasegawa et al., 1996) and to transport serotonin across the cell membrane (Kanner and Bendahan, 1985). Thus, although RBL-2H3 is not of neuronal origin, it is currently the optimal in vitro model system with which to study the serotonin synthesis pathway by biochemical and molecular biological techniques.

In the present study, we hypothesized that the delayed therapeutic action of sertraline may occur via an increase in gene expression of TPH. We demonstrate both in vivo and in vitro that sertraline leads to up-regulation of gene expression and protein levels of TPH as well as to the amount of serotonin available for neurotransmission and that this is mediated, at least in part, by the cAMP/PKA system.

Experimental Procedures

Materials. Male Sprague-Dawley rats (200–225 g) obtained from Asan Institute for Life Science (Seoul, Korea) were used in all experiments. All procedures were approved by the Institutional Animal Care and Use Committee and were performed in compliance with the guidelines set forth by the Laboratory Animal Manual of the Asan Institute for Life Science. Antisera against TPH were obtained from Protos (New York, NY), and cDNA for the determination of rat TPH levels was a generous gift from Dr. Tong H. Joh (Cornell University Medical College, New York, NY). RBL-2H3 cells were purchased from American Type Culture Collection (Manassas, VA). Culture media, Dulbecco's phosphate-buffered saline, streptomycin/penicillin, and fetal calf serum were obtained from Invitrogen (Carlsbad, CA). Proteinase K, phenol, agarose, and Random Prime Labeling Kit were obtained from Roche Molecular Biochemicals (Indianapolis, IN). Sertraline HCl was obtained from Pfizer-Korea (Seoul, Korea). GeneScreen Plus membrane, α-[^{32}P]dCTP, and α-^{35}S-dATP were from PerkinElmer Life Sciences (Boston, MA). N-[2-(p-Bromocinnamylamine)-ethyl]-5-isoquinolinesulfonamide (H-89) was from Seikagaku (Tokyo, Japan), and Elite Kit was from Vector Laboratories (Burlingame, CA). All other chemicals were of reagent grade and were purchased from Sigma (St. Louis, MO) or Merck (Whitehouse Station, NJ).

Animal Treatment. Sertraline was injected i.p. at 10 mg/kg every 24 h for 2 weeks. Control animals were treated with the equivalent volume of the vehicle (0.9% NaCl). The animals were anesthetized with ketamine (75 mg/kg) and xylazine (25 mg/kg) 24 h after the final injection and sacrificed. They were perfused transcardially with saline containing 0.5% sodium nitrite and 10 units/ml heparin sulfate and subsequently with cold 4% formaldehyde (generated from paraformaldehyde) in 0.1 M phosphate-buffered saline (PBS), pH 7.2. Brains were removed and postfixed in the same fixative for 1 h. After being washed in PBS, the tissues were cryoprotected in 30% sucrose at 4°C overnight. Tissues were cut on a cryostat microtome into 40-μm sections and collected in 0.1 M PBS. Of the sections containing the dorsal raphe nucleus (bregma -7.5 to -8.1 mm, according to the atlas of Paxinos and Watson, 1986), every fourth section was subjected to in situ hybridization, and their adjacent sections were subjected to immunocytochemistry.

Immunocytochemistry. After washing in 0.1 M PBS, the sections were incubated in 0.1 M PBS containing 1% bovine serum albumin and 0.2% Triton X-100. After washing in the rinsing buffer containing 0.1 M PBS and 0.5% bovine serum albumin, the sections were incubated with the primary antisera against TPH (1:1000×) overnight at room temperature while being shaken. After being washed in the rinsing buffer, the sections were incubated for 1 h in the biotinylated secondary antisera against rabbit IgG, washed with rinsing buffer, and then further incubated for 1 h in the avidin-biotin complex of the Elite Kit. Antigens were visualized by reaction with 0.05% 3,3′-diaminobenzidine in the presence of 0.003% hydrogen peroxide. After two washes in 0.1 M PBS, the sections were mounted on gelatin-coated slides, dehydrated through graded ethyl alcohols, cleared in xylene, and mounted with a coverslip using Permount (Fisher Scientific, Pittsburgh, PA).

In Situ Hybridization. In situ hybridization was performed essentially as described previously (Hwang et al., 1998). Free-floating 40-μm sections were obtained as described above for immunocytochemistry and placed in vials containing 2× standard saline citrate (SSC; 1× = 0.15 M NaCl and 0.015 M sodium citrate). Tissue sections were prehybridized in 50% formamide, 10% dextran sulfate, 2× SSC, 3.5× Denhardt's solution, 100 mM dithiothreitol, and 1.4 mg/ml denatured salmon sperm DNA for 2 h at 48°C. Rat TPH cDNA (the 548-base pair *Eco*RI-*Eco*RI fragment) (Kim et al., 1991) labeled with ^{35}S-dATP by the random priming method was added to each vial to a concentration of 1 × 10^7 cpm/ml, and hybridization was carried out overnight at 48°C while being shaken. The sections were washed in serial dilutions of SSC at 48°C for 15 min each: 2×, 1×,

$0.5\times$, $0.25\times$, and $0.1\times$ SSC. The sections were then mounted onto gelatin-coated slides in 0.05 M PBS. Air-dried slides were exposed to Kodak XAR-5 film (Eastman Kodak, Rochester, NY) for 1 to 4 days at 4°C to determine the optimal developing time. Slides were subsequently dipped in Kodak NTB-2 emulsion and exposed at 4°C for 1 to 4 weeks. After developing in Kodak D-19, sections were dehydrated, delipidized in ethanol-chloroform (1:1 v/v) for 20 min, counterstained with cresyl violet, and mounted with a coverslip using Permount.

Cell Culture. RBL-2H3 cells were grown in monolayers in minimal essential medium with 2 mM L-glutamine and Earle's balanced salts, 15% fetal calf serum, 100 IU/l penicillin, and 10 μg/ml streptomycin. When confluent, they were subcultured at ratios ranging from 1:3 to 1:5 after dispersing adherent cells with trypsin/EDTA. Cultures were maintained at 37°C in 95% air/5% CO_2 humidified atmosphere. For experiments, cells were plated on polystyrene tissue culture dishes at a density of 3×10^6 cells/60-mm plate. After 24 h, cells were fed with fresh media, which was considered time 0.

RNA Isolation and Northern Analysis. Total RNA was isolated from the cultured cells as described previously (Hwang and Choi, 1995) and was denatured and resolved in 1% agarose gel containing 6% formaldehyde in the running buffer (20 mM MOPS, pH 7.0, 5 mM sodium acetate, and 1 mM EDTA). The RNA was then transferred to GeneScreen Plus membrane in $10\times$ SSC. The membranes were baked at 80°C for 2 h and prehybridized in 50% formamide, 1% SDS, 1 M NaCl, and 10% dextran sulfate at 42°C for at least 15 min. Hybridization was performed by adding saturating amounts of ^{32}P-labeled and denatured cDNA probe (the 548-base pair EcoRI-EcoRI fragment of rat TPH) (Kim et al., 1991) along with 100 μg/ml of denatured salmon sperm DNA and incubating at 42°C overnight while being shaken. The membranes were washed twice in the solution containing $1\times$ SSC and 1% SDS at 65°C for 30 min followed twice in $0.1\times$ SSC at room temperature for 30 min. Autoradiography was performed overnight at −80°C.

Immunoblot Analysis. Immunoblot analysis was performed as described previously (Hwang et al., 1999). Cells were washed twice, harvested, and homogenized in 5 mM potassium phosphate, pH 6.5, containing 0.2% Triton X-100. After protein separation by SDS-polyacrylamide gel electrophoresis and electrophoretic transfer to a Nytran filter, the filter paper was incubated in blocking buffer containing 1% gelatin, 150 mM NaCl and 50 mM Tris-Cl, pH 7.4, for 2 h at room temperature. The blots were then treated with TPH antisera diluted to 1:1000× in rinsing buffer (0.25% gelatin, 5% goat serum, 0.05% Nonidet P-40, 5 mM EDTA, 150 mM NaCl, and 50 mM Tris-Cl, pH 7.4) for 2 h. After thorough rinsing with rinsing buffer, the blots were incubated with horseradish peroxidase–conjugated goat anti-rabbit IgG diluted to 1:200 in rinsing buffer. The antigen-antibody complexes were visualized by reaction with 0.05% 3,4-diaminobenzidine containing 0.05% hydrogen peroxide.

Determination of TPH Enzyme Activity. Cells were washed twice, harvested, and homogenized in 5 mM potassium phosphate, pH 6.5, containing 0.2% Triton X-100, and TPH activity was measured as described previously (Vrana et al., 1993). Briefly, the cell homogenate was mixed with the same volume of reaction mixture containing 0.05 mM tryptophan, 50 mM HEPES, pH 7.0, 5 mM dithiothreitol, 0.01 mM $Fe(NH_4)_2(SO_4)_2$, 0.5 mM 6-MPH_4, 0.1 mg/ml catalase, and 1 μCi of [^{3}H]tryptophan and incubated at 37°C for 15 min. After unreacted tryptophan is adsorbed with acid-washed charcoal and centrifugation, the [^{3}H]H_2O in the supernatant was measured by liquid scintillation. The amounts of protein were measured by use of the method described by Bradford (1976).

Determination of Serotonin Levels. For the determination of released serotonin, the culture medium was collected and treated with perchloric acid to a final concentration of 0.4 M and centrifuged. For the determination of intracellular serotonin, cells were washed twice and harvested in 1 ml of Dulbecco's phosphate-buffered saline. After pelleting and homogenization in perchloric acid (0.4 M), the acid-soluble fraction was diluted appropriately. Both samples were

then subjected to high-performance liquid chromatography using a C18 column (Novapak Corporation, Eatontown, NJ) in the mobile phase consisting of 0.1 M citric acid, 0.1 M sodium acetate, pH 4.1, 0.2 mM EDTA, and 5% ethanol, and serotonin was detected electrochemically by a Waters 460 detector (Waters, Milford, MA). The amount of serotonin was calculated using Waters 991 computerized integrator system, and a curve was prepared every time. Background serotonin in the culture medium was subtracted from all control and experimental values for released serotonin. Total amount of serotonin was calculated as the sum of the intracellular and released serotonin. Amount of protein was determined by use of the method described by Bradford (1976).

Data and Image Analyses. Statistical analyses and comparisons were made using analysis of variance and Newman-Keuls multiple comparisons test, and $p < 0.05$ was considered statistically significant. For image analyses, signal intensities were determined densitometrically using a computer-assisted image analysis system (Quantity One, version 4.2.1; Bio-Rad, Hercules, CA). Northern and immunoblot analyses were performed on three independent culture preparations, and data are expressed as mean ± S.D. values calculated by use of the following equations: (densitometric value for each treatment condition / 28S) / (densitometric value for untreated control / 28S) and (densitometric value for each treatment condition / protein amount) / densitometric value for untreated control/protein amount), respectively. For the determination of TPH activity and serotonin, experiments were performed in duplicate on triplicate samples for each condition and were repeated on three different culture preparations.

Results

Sertraline Up-Regulates TPH Gene Expression In Vivo. To test our hypothesis that sertraline may lead to changes in gene expression of the serotonin synthesis enzyme TPH, in vivo experiments were first carried out. Rats were treated with sertraline by daily intraperitoneal injection at 10 mg/kg for 2 weeks and were compared with vehicle-injected control animals ($n = 10$ in each group). In situ hybridization for TPH was performed on brain sections of the dorsal raphe nucleus.

Figure 1 shows typical photomicrographs of the dorsal raphe nucleus sections of the sertraline-treated and vehicle-treated control animals. Although a similar pattern of TPH expression was observed in the two groups, the dorsal raphe
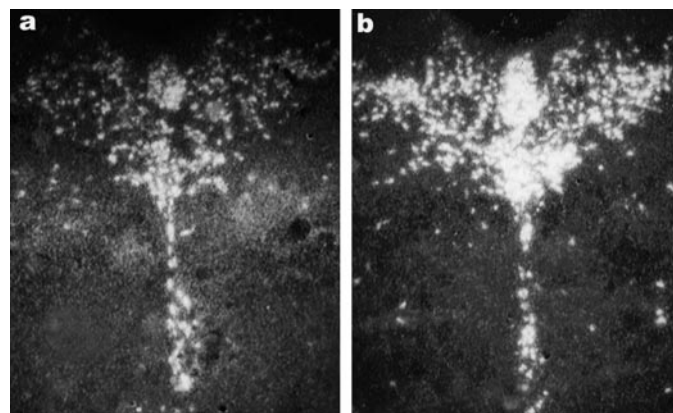

Fig. 1. Typical photomicrographs of the dorsal raphe nucleus showing up-regulation of TPH mRNA level by sertraline. Rats were treated daily with (a) vehicle or (b) sertraline for 2 weeks, and the dorsal raphe sections were subjected to in situ hybridization to determine TPH signals. Densitometric analysis of sections of the dorsal raphe between bregma −7.5 to −8.1 mm revealed a 3.4 ± 1.2-fold increase ($n = 10$ each, $p < 0.05$) in the sertraline-treated animals.

section of the sertraline-treated animal exhibited a dramatically higher TPH mRNA signal. Consistent increases were shown in all sections of the dorsal raphe between bregma -7.5 to -8.1 mm. Densitometric analysis performed on every fourth section of the dorsal raphe revealed a 3.4 ± 1.2-fold increase in the sertraline-treated animals compared with the control animals ($p < 0.05$).

Sertraline Up-Regulates TPH Protein Level In Vivo. To test whether this change in the level of TPH mRNA was reflected in the amount of TPH protein, the adjacent sections of dorsal raphe of the animals in Fig. 1 were subjected to immunocytochemistry. As shown in Fig. 2, the TPH-positive cells of the sertraline-treated animals showed more intense immunoreactivity than the vehicle-treated control animals. Densitometric analysis of the sections between bregma -7.5 and -8.1 revealed a 1.5 ± 0.2-fold increase in the sertraline-treated animals compared with the control animals ($p < 0.05$).

Sertraline Up-Regulates TPH Gene Expression In Vitro. To more quantitatively assess the effects of sertraline on the serotonin synthesis, we turned to an in vitro system so that the effect of a single agent could be studied in an isolated system. RBL-2H3 cells were first treated with various concentrations of sertraline for 24 h, and the degree of TPH gene expression was compared with that of untreated control animals by Northern blot analysis.

Figure 3a illustrates a typical Northern blot showing two TPH mRNA species of 1.8 and 4.0 kilobases, consistent with those found in the central nervous system (Kim et al., 1991), with the smaller form present in higher abundance. The sertraline treatment enhanced TPH gene expression in a dose-dependent manner. Densitometric analysis (Fig. 3b) revealed that 10 μM sertraline caused maximal increases in the smaller and larger transcripts to 8.11 ± 1.24- and 3.73 ± 0.73-fold of that of untreated control animals, respectively. Higher concentrations of sertraline did not further increase the TPH mRNA levels (data not shown). Thus, 10 μM was considered to be the optimal concentration and was used in all subsequent in vitro experiments.

To determine the time course of the sertraline effect, the cells were treated with 10 μM sertraline for 0, 6, 24, 48, and 72 h, and the amounts of TPH mRNAs were assessed by Northern blot analysis. As shown in Fig. 4a, TPH mRNAs

levels increased within 6 h and continued to increase up to 24 h. This induced level was maintained up to 48 h. Densitometric analysis (Fig. 4b) revealed that after 6 h, the small TPH transcript had already accumulated to 5.09 ± 0.59-fold of that of the untreated control animals. Even after 72 h, its level was still higher than that of the untreated control animals (3.53 ± 0.51-fold).

Sertraline Up-Regulates TPH Protein Level In Vitro. Whether the TPH protein level was also augmented by sertraline was tested by immunoblot analysis using the specific antisera against TPH. The cells were treated with 10 μM sertraline for various lengths of time, and the cell lysates were subjected to immunoblot analysis. The result of a typical experiment is shown in Fig. 5. A single immunopositive band of approximately 51 kDa was detected, in agreement with the size of rat TPH protein reported by others (Kim et al., 1991). Both 48- and 72-h treatments led to up-regulation of TPH protein to approximately 2.5-fold of that of the untreated control animals. Thus, parallel increases in both TPH mRNA and protein by sertraline were observed in vitro as well.
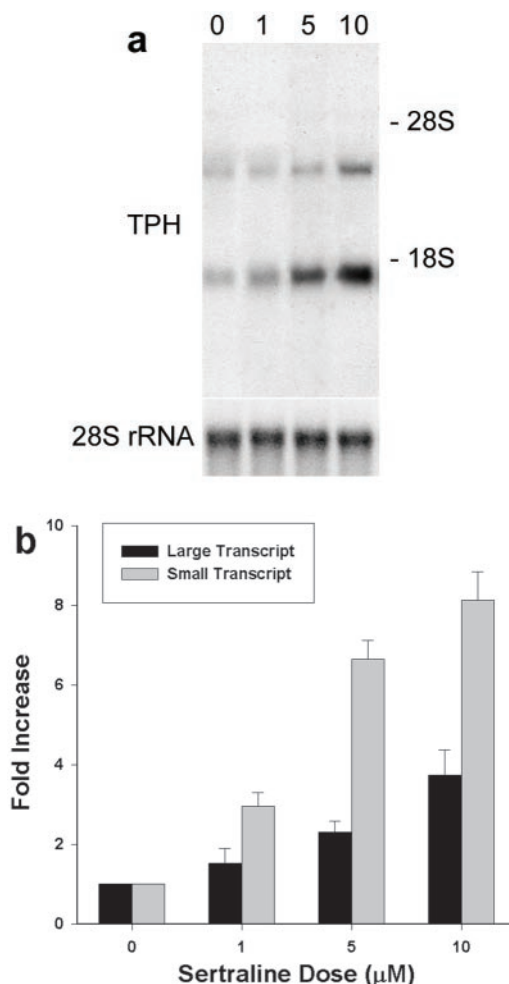
Fig. 3. Northern blot analysis showing up-regulation of the TPH mRNA level by sertraline in RBL-2H3 cells. Cells were treated with 0, 1, 5, or 10 μM sertraline for 24 h. a, a typical Northern blot analysis. Total RNA was subjected to Northern blot analysis using a cDNA for rat TPH (top) and compared with 28S rRNA band (bottom); b, densitometric analyses of the bands. All values for sertraline-treated samples were significantly different ($p < 0.05$) from those of untreated control animals except for the large transcript at 1 μM.
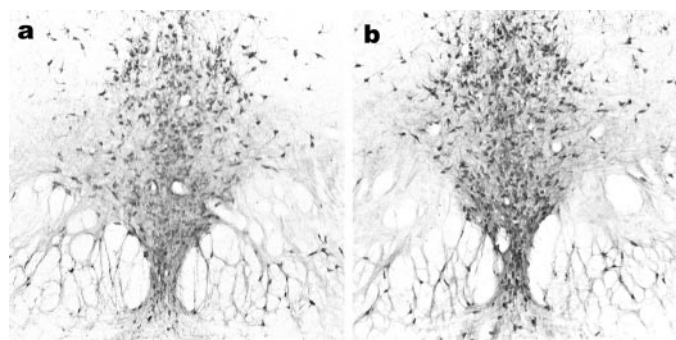
Fig. 2. Typical photomicrographs of the dorsal raphe nucleus showing up-regulation of the TPH protein level by sertraline. Rats were treated daily with (a) vehicle or (b) sertraline for 2 weeks, and the dorsal raphe sections were subjected to immunocytochemistry against TPH. Densitometric analysis of sections of the dorsal raphe between bregma -7.5 to -8.1 mm revealed a 1.5 ± 0.2-fold increase ($n = 10$ each, $p < 0.05$) in the sertraline-treated animals.

Sertraline Up-Regulates TPH Enzymatic Activity In Vitro. Because changes in catalytic activity of TPH would ultimately determine the rate of sertraline synthesis, it was important to test whether the up-regulation of TPH protein by sertraline would result in increased enzyme activity. For this, we determined the time course of changes in TPH activity after sertraline treatment. Lysates of cells exposed to 10 μM sertraline for various lengths of time were subjected to TPH assay. As shown in Fig. 6, the enzyme activity began to increase after 24 h of the sertraline treatment. The maximal effect was observed at 48 and 72 h with 2.5-fold up-regulation.

Sertraline Increases Serotonin Levels In Vitro. Whether these increases in TPH gene expression and activity would ultimately lead to changes in serotonin availability was tested. For this, the cells were treated with 10 μM sertraline for various periods of time, and the amounts of

intracellular and extracellular serotonin were determined. As shown in Fig. 7a, extracellular serotonin increased dramatically in the presence of the serotonin uptake blocker, as would be expected. In 96 h, the amounts of released serotonin of the treated and untreated cells were 140 ± 11 and 39 ± 6 nmol/mg protein, respectively, showing a 3.6-fold increase. The intracellular serotonin concentration, on the other hand, decreased with time, up to 48 h (Fig. 7b), probably because of the cells' inability to take up the neurotransmitter through the transporter. Subsequently, the intracellular serotonin gradually increased, probably because of the increase in serotonin synthesis rate and, consequently, the total serotonin, determined as the sum of the intracellular and extracellular serotonin, became higher than that in the untreated control animals (Fig. 7c). At 96 h, the total serotonin amounts of the
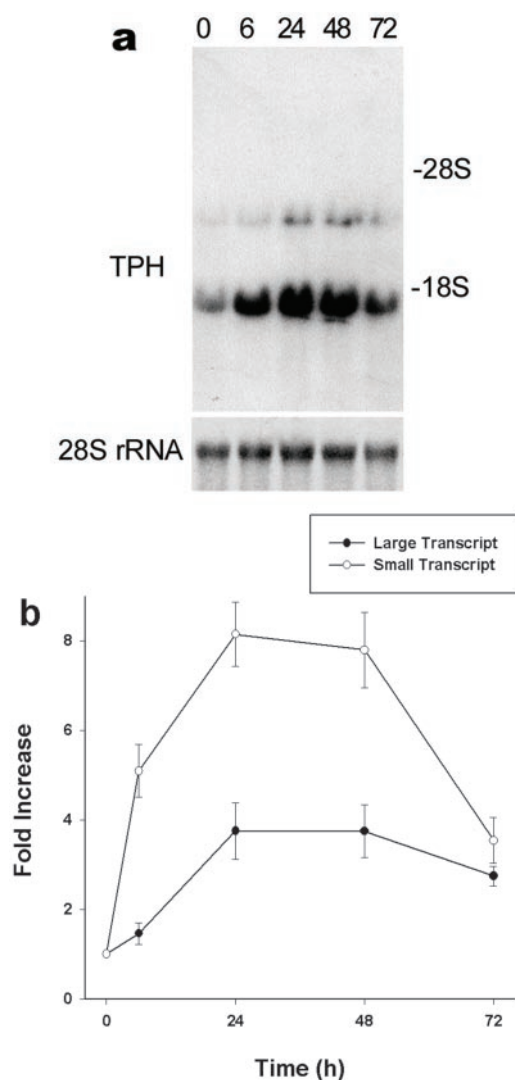

Fig. 4. Northern analysis showing time course of TPH up-regulation by sertraline. Cells were treated with 10 μM sertraline for 0, 6, 24, 48, and 72 h, and the amounts of TPH mRNAs were assessed by the use of Northern blot analysis. a, a typical Northern blot analysis. Total RNA was subjected to Northern blot analysis using a cDNA for rat TPH (top) and was compared with 28S rRNA band (bottom); b, densitometric analyses of the bands. All values for sertraline-treated samples were significantly different ($p < 0.05$) from those for untreated control animals except for the large transcript at 6 h.
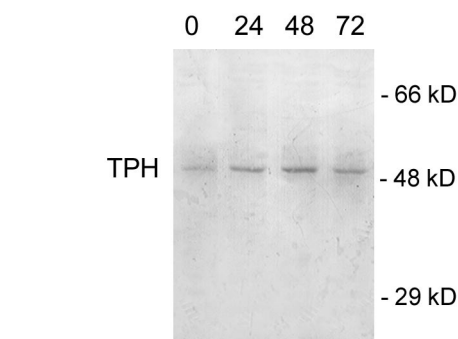

Fig. 5. Effect of sertraline on the TPH protein level. Cells were exposed to 10 μM sertraline for 24, 48, and 72 h, and the cell lysates were subjected to immunoblot analysis against TPH. Experiments were performed on three independent culture preparations, and the results of a typical experiment are shown. The TPH protein level was up-regulated by 24-, 48-, and 72-h sertraline treatments to 1.78 ± 0.16-, 2.53 ± 0.22-, and 2.50 ± 0.19-fold of that of the untreated control, respectively. All values for sertraline-treated samples were significantly different ($p < 0.05$) from those for untreated control animals.
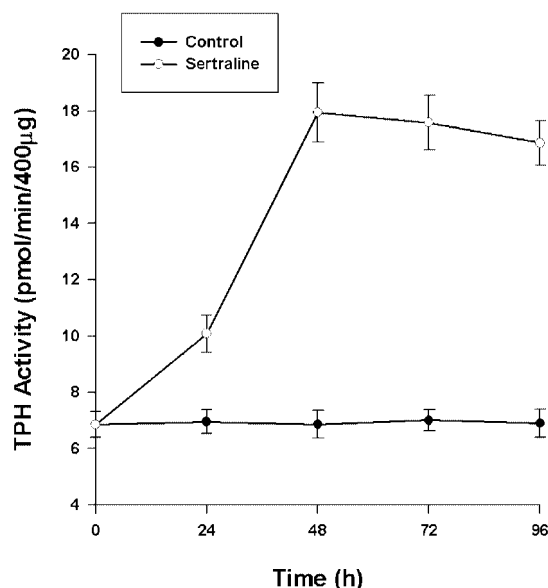

Fig. 6. Effect of sertraline on TPH enzyme activity. Cells were cultured in the presence or absence of 10 μM sertraline for 24, 48, 72, and 96 h, and TPH activity was measured by the radioenzymatic method as described under *Experimental Procedures*. Data given are mean $\pm$ S.D. values expressed as picomoles of product per minute per 400 μg of protein. All values for the sertraline-treated samples were significantly different ($p < 0.05$) from those for untreated control animals.
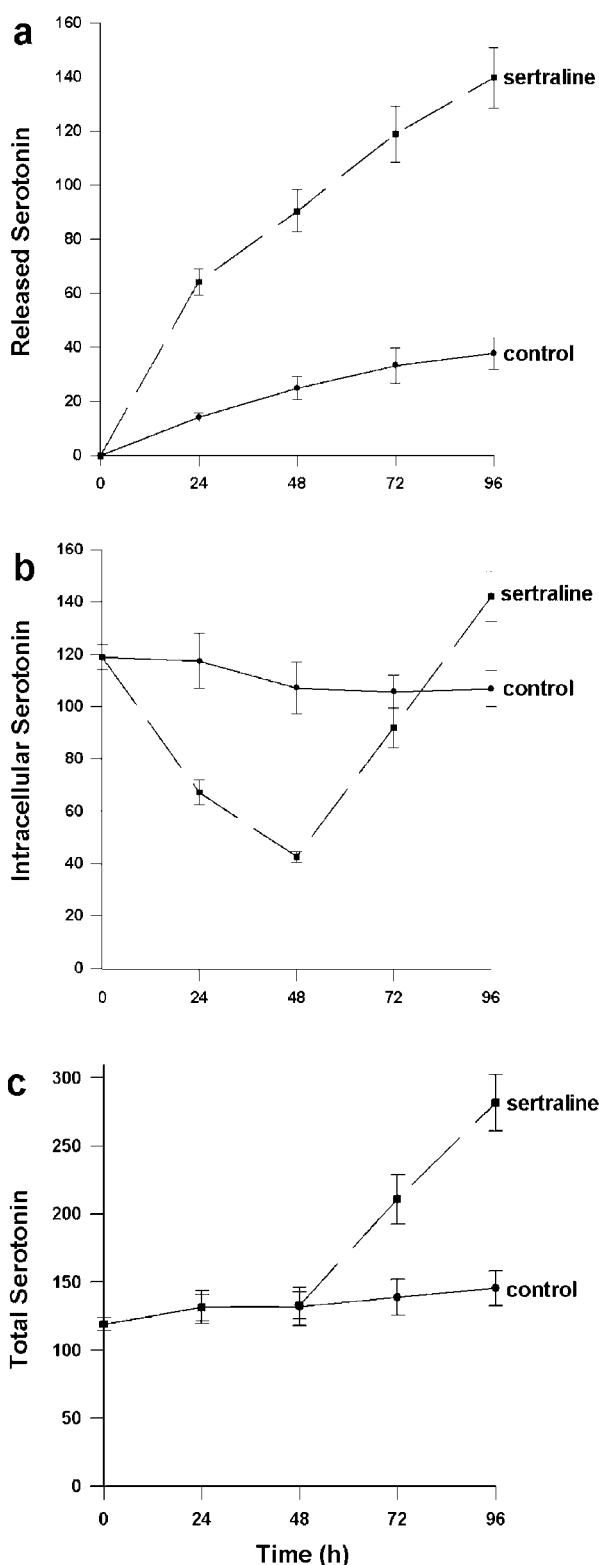
Fig. 7. Effect of sertraline on serotonin levels. Cells were cultured in the presence or absence of 10 μM sertraline for 24, 48, 72, and 96 h, and the amounts of serotonin were measured by high-performance liquid chromatography coupled with an electrochemical detector. Released serotonin (a), intracellular serotonin (b), and total serotonin (c) were calculated by adding intracellular and extracellular serotonin values for each time point. Data given are mean $\pm$ S.D. values expressed as nmol serotonin/mg protein. All values were significantly different ($p < 0.05$) from those for untreated control animals except for intracellular serotonin at 72 h and total serotonin at 24 and 48 h.

sertraline-treated and -untreated cells were 281 ± 20 and 145 ± 13 nmol/mg protein, respectively, showing a 94% increase.

Fluoxetine Also Up-Regulates TPH Expression. To begin to assess whether the up-regulation of serotonin synthesis system might be a general phenomenon for the SSRIs, we asked whether fluoxetine, another commonly used SSRI, might also have similar effects on TPH gene expression. RBL-2H3 cells were treated for 24 h with fluoxetine or, for comparison, the non-SSRI antidepressant chlorpromazine. Changes in the TPH gene expression level were monitored by Northern blot analysis. Densitometric analysis of the Northern blot showed that fluoxetine at 10 μM effectively up-regulated the levels of the small and large TPH transcripts by 9.32 ± 1.54- and 3.89 ± 0.85-fold ($p < 0.05$ versus untreated control), respectively. On the other hand, chlorpromazine was ineffective and resulted in no significant changes (1.10 ± 0.52- and 1.05 ± 0.41-fold, respectively; $p > 0.05$).

Protein Kinase A Is Involved in the Sertraline-Induced TPH Gene Expression. Because cAMP causes the induction of TPH gene expression, it was possible that cAMP might mediate the sertraline effect on TPH gene expression. To test this, we determined whether PKA inhibition might attenuate the TPH up-regulation by sertraline. The cells were treated with sertraline in the absence or presence of the selective PKA inhibitor H-89 (Chijiwa et al., 1990; Hwang et al., 1997), and Northern blot analysis was performed. As shown in Fig. 8a, the sertraline-induced up-regulation of TPH gene expression was attenuated. Densitometric analysis showed (Fig. 8b) that the inhibition of PKA resulted in the up-regulation of both the small and large transcripts to only 53% and 58% of sertraline alone, respectively.

Discussion

Most drugs that are used to treat depression are known to interact with the serotonin system. However, because the serotonin terminals innervate almost all regions of the brain and a number of serotonin receptor subtypes exist, the mechanisms of action of these drugs have been difficult to understand.

In the present study, we demonstrated in vivo and in vitro that sertraline augments serotonin synthesis via the up-regulation of gene expression and protein levels of TPH, the rate-limiting enzyme in the serotonin synthesis pathway. The increase in both intracellular and released serotonin by sertraline observed in this study may provide insight into the mechanism by which long-term sertraline administration gives rise to the augmentation of synaptic serotonin level. We observed the changes in situ by the use of both immunocytochemistry and in situ hybridization. This was further corroborated by the results of immunoblot and Northern blot analyses obtained from in vitro studies.

Daily administration of sertraline for 2 weeks led to an increase in the TPH mRNA and protein levels in the serotonergic neurons of the dorsal raphe nucleus (Figs. 1 and 2). This was corroborated by our in vitro data (Figs. 3–5) obtained from RBL-2H3. Although both in vivo and in vitro data revealed up-regulation of TPH by sertraline, the changes were more dramatic in vitro. This is probably because the in vitro condition was optimized for dose and duration of treatment, whereas only one regimen was followed

in vivo. It is possible that we may have missed the time point at which the maximal effect was reached in the animals. Although we assessed changes after a 2-week treatment, at which time therapeutic improvement is observed in patients with depression, it is possible that rat and humans differ in their time course of sertraline efficacy. Thus, under optimal conditions with appropriate time and dose, more dramatic changes might be attained in vivo as well.

The increases in the TPH protein and activity levels were not as dramatic as that in the transcript level both in vivo and in vitro. The mRNA level was maximally induced to approximately 8-fold in the presence of 10 μM sertraline (Figs. 3 and 4), whereas the same dose resulted in the protein level to be maximally increased only to 2.5-fold (Fig. 5). Although it is difficult to explain its mechanism, such a phenomenon seems to be common to the enzymes in the monoamine synthesis pathway, as we have noted previously (Hwang et al., 1994; Hwang and Choi, 1995).

The increases in TPH gene expression and activity were ultimately accompanied by an increase in the availability of

Fig. 8. Effect of PKA inhibition on the TPH up-regulation by sertraline. Cells were exposed to 10 μM sertraline for 24 h in the presence or absence of the PKA inhibitor H-89 (30 μM). a, a typical Northern blot analysis. Total RNA was subjected to Northern blot analysis using a cDNA for rat TPH (top) and was compared with the 28S rRNA band (bottom); C, control; S, sertraline; H, H-89. b, densitometric analyses of the bands. Values for the sertraline-plus-H-89–treated sample were significantly different ($p < 0.05$) from the sertraline-treated and H-89–treated samples.

serotonin. The temporal differences for the maximal responses among TPH mRNA, protein, and activity levels and serotonin amount seem to be caused by the time required for their processing. Translation of the TPH protein and the subsequent increase in the amount of the total TPH protein pool seems to take approximately 24 h, derived from the peak time differences for TPH mRNA and protein, as determined by Northern and immunoblot analyses, respectively. On the other hand, the increases in TPH protein and activity levels were temporally similar, suggesting that the newly produced TPH protein seemed to be incorporated into the catalytically active pool of the enzyme rather rapidly. The up-regulation of the total serotonin pool temporally followed the increase in TPH activity by a lag of approximately 24 h. Such a temporal relationship among changes in the levels of transcript, protein, and functional activity has been demonstrated previously for the monoamine synthesis pathways (Hwang et al., 1994, 1997; Hwang and Choi, 1995).

From our data, it is possible to speculate that in patients with depression, sertraline may ultimately lead to the augmentation of gene expression of TPH and consequently of the rate of serotonin synthesis. The fact that the SSRI fluoxetine, but not the non-SSRI chlorpromazine, also exerts similar effect on TPH expression suggests that this mechanism may be shared by other SSRIs. However, a more extensive comparison of the action mechanisms for various other SSRIs and non-SSRIs would be required before a definitive conclusion could be drawn.

Long-term antidepressant administration has been shown to lead to PKA nuclear translocation (Nestler et al., 1989) and increased expression of cAMP-response element-binding protein (Nibuya et al., 1996). The human TPH contains a cAMP-responsive element in its promoter (Boularand et al., 1995), and its gene expression (Foguet et al., 1993; Florez and Takahashi, 1996; Green et al., 1996) and enzyme activity (Florez and Takahashi, 1996; Valenciano et al., 2000) are up-regulated by cAMP. We show in the present study that the sertraline-induced TPH up-regulation is attenuated by the PKA inhibitor H-89. Taken together, it is possible that TPH gene is a target for the cAMP/PKA system activated by long-term sertraline administration. Because H-89 did not completely block the TPH induction by sertraline, however, it can be speculated that additional mechanisms may also participate in this regulatory action.

Decreased PKA activity has been observed in patients with major depression (Manier et al., 1996; Shelton et al., 1999; Perez et al., 2001). Hence, it can be proposed that patients with depression may suffer from low TPH activity and gene expression because of low PKA activity, and the pharmacological action of sertraline is achieved by activation of PKA, leading to the up-regulation of TPH and eventually the enhancement of the serotonin neurotransmission.

One of the problems of the currently prescribed antidepressant drugs is that the delay in onset of action often contributes to the high suicide rate (15%) of patients with depression (Akiskal, 1995). Thus, the development of drugs with similar efficacy but faster action would be highly beneficial (Blier, 2001). Conceivably, drugs that modulate the serotonin synthesis via a signal transduction pathway acting downstream of the uptake inhibition, such as the cAMP/PKA system, may provide an alternative and faster therapeutic method. As a corollary, delineation and identification of sub-
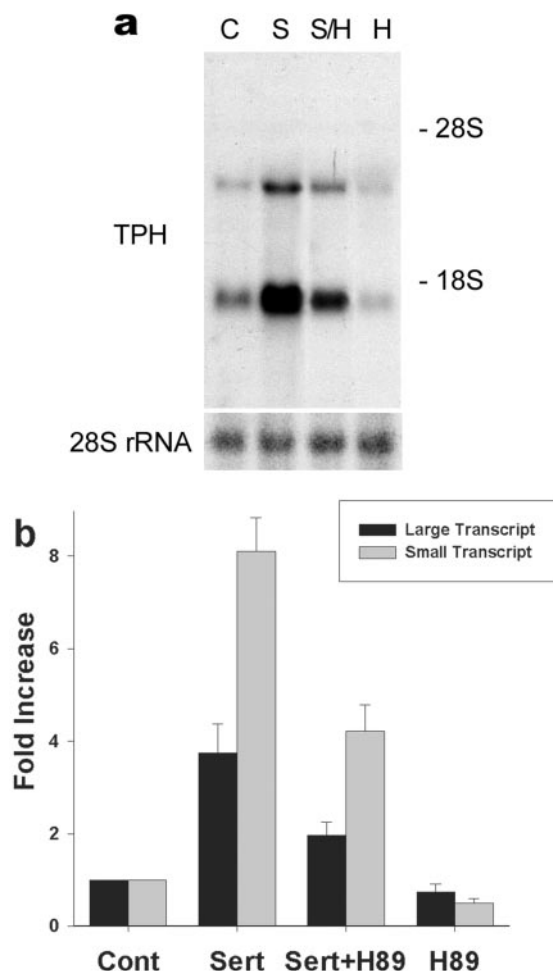
types of the receptors and enzymes involved in the signaling pathway leading to the cAMP increase by sertraline may provide insight into the development of such drugs.

In conclusion, we demonstrate that, in addition to the short-term action of reuptake inhibition of serotonin, sertraline also has a long-term effect on the serotonin neurotransmission via the elevation of gene expression and protein level of the serotonin- synthesizing enzyme TPH, mediated at least partly by cAMP. This may be one of the mechanisms for the pharmacological action of sertraline in antidepressive therapy.

Acknowledgments

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