Polarized Distribution of Interleukin-1 Receptors and Their Role in Regulation of Serotonin Transporter in Placenta

ABSTRACT

We investigated the expression of interleukin-1 (IL-1) receptors and their involvement in the regulation of the serotonin transporter gene expression in human placenta. IL-1β is an activator of the serotonin transporter gene expression in JAR human placental choriocarcinoma cells as demonstrated by an increase in the steady-state levels of the transporter mRNA and in serotonin transport activity. This activation is blocked by IL-1 receptor antagonist. Genistein also blocks the effect of IL-1β, indicating involvement of tyrosine phosphorylation in the process. Treatment of JAR cells with IL-1β activates mitogen-activated protein kinases and nuclear factor-κB. The nuclear factor-κB that is responsive to IL-1β in these cells is the p65 homodimer. Northern blot analysis and reverse transcription-polymerase chain reaction revealed that JAR cells and human placenta express type I and type II IL-1 receptors. The binding sites for IL-1α and IL-1β are localized predominantly in the maternal-facing brush border membrane of the syncytiotrophoblast. These results show that IL-1 in the maternal circulation is likely to play a critical role in the regulation of the serotonin transporter gene expression in the placenta.

In humans, the serotonin transporter (SERT) is expressed in the plasma membrane of serotonergic neurons, platelets, and placenta (Rudnick and Clark, 1993; Ganapathy and Leibach, 1995). Molecular cloning studies have established that the transporter expressed in these tissues is identical, encoded by the same gene (Ramamoorthy et al., 1993a; Lesch et al., 1993). In serotonergic neurons, the transporter functions in the clearance of serotonin from the synaptic cleft by active reuptake of the neurotransmitter into the presynaptic neurons. In platelets, the role of the transporter is to take up serotonin from the circulation and accumulate it inside the cells. In contrast, the function of the transporter in the placenta has not yet been established despite the fact that this tissue expresses very high levels of the transporter as evidenced from serotonin transport activity in maternal-facing brush border membrane vesicles (Balkovetz et al., 1989). Speculations have been made, however, that the transporter in the placenta may be involved in the clearance of serotonin, a potent vasoconstrictor, from the intervillous space and that such a process may be essential to maintain the uteroplacental blood flow (Ganapathy and Leibach, 1994, 1995). The findings that the placental SERT is inhibited by cocaine and amphetamines (Prasad et al., 1994; Ramamoorthy et al., 1995a) also have led to the speculation that interference with the transporter-mediated placental clearance of serotonin by these drugs may be involved in the pathogenesis of clinical symptoms in the mother and the developing fetus known to be associated with the maternal abuse of these drugs during pregnancy.

Human placental choriocarcinoma cells express SERT (Cool et al., 1991; Jayanthi et al., 1994). To date, these are the only cell lines of human origin that constitutively express SERT. These cell lines have proved to be very useful in studies relating to the regulatory aspects of the transporter. These studies have shown that the transporter is up-regulated by cAMP (Cool et al., 1991; Ramamoorthy et al., 1993b), staurosporine (Ramamoorthy et al., 1995b), herbimycin A (Prasad et al., 1997), and epidermal growth factor (Kekuda et al., 1997) by increasing the steady-state levels of the transporter mRNA. Calmodulin has been shown to increase the transporter activity, but this effect appears to be at the post-translational level (Jayanthi et al., 1994). More recently, interleukin-1β (IL-1β) was found to enhance SERT activity and this effect was associated with an increase in the steady-state levels of the transporter mRNA and protein (Ramamoorthy et al., 1995c). There was also evidence indicating that the process involved up-regulation of the transporter gene expression at the transcriptional level. Even though the
IL-1β is the first cytokine known to up-regulate SERT gene expression. The regulation of the transporter expression by IL-1β is likely to have profound clinical relevance with respect to not only placental function but also serotonergic neurotransmission. The present investigation was undertaken to study the expression and distribution of IL-1 receptors in choriocarcinoma cells and in normal placenta and also to study the signaling pathways associated with the IL-1β-dependent up-regulation of SERT gene expression. The results of this investigation show that 1) normal placenta and JAR choriocarcinoma cells express both type 1 and type II IL-1 receptors; 2) the receptors in normal placenta exhibit a polarized distribution, being present predominantly in the maternal-facing brush border membrane of the syncytiotrophoblast; and 3) up-regulation of SERT gene expression by IL-1β in JAR cells is associated with the activation of mitogen-activated protein (MAP) kinases and nuclear factor-κB (NF-κB) and is inhibited by IL-1 receptor antagonist.

**Experimental Procedures**

**Materials.** The JAR human placental choriocarcinoma cell line was purchased from the American Type Culture Collection (Rockville, MD). Culture media (RPMI 1640), penicillin, and streptomycin were obtained from Life Technologies, Inc., Rockville, MD. 5-[1,2-3H]Hydroxytryptamine (serotonin) (sp. radioactivity, 26.3 Ci/mmol) was obtained from DuPont-NEN. [γ-32P]ATP, [α-32P]dCTP, and [α-32P]cytidine 5′-triphosphate, and [32P]NAI were obtained from Amersham (Arlington Heights, IL). IL-1β and IL-1 receptor antagonist (IL-1Ra) were purchased from Bachem (Torrance, CA). Protein A-agarose and myelin basic protein were obtained from Sigma Chemical Co., St Louis, MO. NF-κB consensus oligonucleotide was obtained from Promega, Madison, WI. Anti-ERK antibody and anti-p50, anti-p52, anti-p65, and anti-c-Rel were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The culture medium (RPMI 1640) was purchased from Mediatech (Herndon, VA). The hormones used for supplementation of the culture medium were from Sigma Chemical Co.

**Cell Culture.** JAR cells were cultured as described previously (Cool et al., 1991). Treatment with different reagents was carried out for indicated time periods in a hormonally defined medium before transport measurements. The defined medium consisted of RPMI 1640, supplemented with insulin (5 μg/ml), apotransferrin (5 μg/ml), proglandin E2 (2.5 × 10^-9 mg/ml), and thyroxine (5 × 10^-12 M).

**Transport Measurements in Cells.** The dishes containing monolayer cultures of the cells were taken out of the incubator and left standing at room temperature for 2 hr. The culture medium was then aspirated, and the cells were washed once with the transport buffer. One milliliter of transport buffer containing radiolabeled serotonin was added to the cells and incubated for 3 min at room temperature. Transport was terminated by aspirating the buffer and subsequently washing the cells three times with fresh transport buffer. The cells were lysed with 1 ml of 0.2 N NaOH/1% SDS and the lysate transferred to scintillation vials for quantitation of radioactivity. The composition of the transport buffer was 25 mM HEPES-Tris, pH 7.5, 140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl2, 0.8 mM MgSO4, 5 mM glucose, and 0.1 mM iproniazid, an inhibitor of monoamine oxidases. Serotonin transport that occurred independent of SERT was determined by measuring the transport in the presence of 0.1 mM imipramine, and this component was always <10% of the total transport measured in the absence of imipramine.

**Northern Blot Analysis of SERT mRNAs.** Poly(A)1 RNA was prepared from JAR cells cultured under different conditions with the FastTrack mRNA isolation kit (Invitrogen). Total RNA was isolated from human term placentas with Ce-trifluorocetate isopicnic centrifugation according to the

**MAP Kinase Assay.** JAR cells were treated with 10 ng/ml IL-1β in plain RPMI medium for different time points. MAP kinase assays were performed as described by Scherle et al. (1997). Control and IL-1β-treated cells were washed twice with ice-cold PBS. Cells were lysed with lysis buffer (PBS, containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mg/ml PMSF, 0.15 to 0.3 trypsin inhibition unit/ml aprotinin, and 1 mM sodium orthovanadate) and the lysates were precleared with 1 μg of normal rabbit IgG and 20 μl of protein A-agarose conjugate. Cell extracts were treated with 1 μg of anti-extra cellular signal-regulated kinase (ERK) antibody for 1 h at 4°C. Then 30 μl of protein A-agarose conjugate was added and incubated at 4°C overnight. The immunoprecipitate was washed with lysis buffer four times and once with 20 mM HEPESTris, pH 7.0. MAP kinase activity was assayed by resuspending the immunoprecipitate in kinase assay buffer that contained 20 mM HEPES, 5 mM 2-mercaptoethanol, 10 mM MgCl2, 0.1 mg/ml BSA, 10 μM ATP, 10 μCi [γ-32P]ATP, and 2.5 μg of myelin basic protein and incubated at 30°C for 15 min. Reaction was stopped with SDS loading buffer and phosphorylation of myelin basic protein was examined on a 14% SDS-polyacrylamide gel. The gel was dried and subjected to autoradiography.

**Preparation of the Nuclear Extract.** Nuclear extracts were prepared according to the method of Ledebur and Parks (1995). JAR cells were treated with 10 ng/ml IL-1β for different time periods. The cells were washed twice with ice-cold PBS. Cells were lysed in a hypotonic buffer that contained 10 mM HEPES/Tris, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM PMSF, 0.5 mM dithiothreitol, 10 μg/ml leupeptin, and 0.5% Nonidet P-40 for 10 min on ice. The lysates were centrifuged at 12,000 g for 10 min. The pelleted nuclei were resuspended in a high salt lysis buffer (20 mM HEPES, pH 7.9, 25% glycerol, 720 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM dithiothreitol, and 10 μg/ml leupeptin) on ice for 15 min. The lysed nuclei were spun at 12,000 g for 10 min. The supernatant was mixed with four volumes of storage buffer (20 mM HEPES/Tris, pH 7.9, 20% glycerol, 50 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM dithiothreitol, and 10 μg/ml leupeptin). The nuclear extract was stored at -70°C in small aliquots until used.

**Electrophoretic Mobility Shift Assay (EMSA).** NF-κB binding consensus oligonucleotide had the sequence 5′-AGT TGA GGG GAC TTT CCC AGG-3′. The oligonucleotide was labeled with [γ-32P]ATP with T4 polynucleotide kinase and purified with Sephadex G50 gel filtration chromatography. The DNA binding was done in 20 μl of binding buffer that contained 250 mM NaCl, 20 mM KCl, 10 mM HEPES, pH 7.5, 1.1 mM EDTA, 1.0 mM 2-mercaptoethanol, 10.5% glycerol, 0.1 μg/μl poly dI/dC, 0.15 mM MgCl2, and 0.5 × 105 cpm 32P-labeled probe at room temperature for 30 min. In experiments addressing the specificity of the electrophoretic mobility shift, 3.5 pmol of unlabeled oligonucleotide was added to compete with 32P-labeled oligonucleotide. Samples were electrophoresed on a 4% acrylamide with 0.5 × 45 mM Tris-borate, pH 8.0, containing 1 mM EDTA as the running buffer. Whenever unlabeled oligonucleotides were used for competition, they were treated with the nuclear extract for 10 min at room temperature before the addition of the probe. For the supershift assays, the nuclear extracts were treated with 1.5 μg of the primary antibodies against NF-κB p65, NF-κB p50, NF-κB p52, and NF-κB c-Rel for 20 min at room temperature before the addition of the labeled probe.

**Northern Blot Analysis of IL-1 Type I and IL-1 Type II mRNAs.** Poly(A)+ mRNA samples isolated from the JAR human placental choriocarcinoma cells with the FastTrack mRNA isolation kit (Invitrogen). Total RNA was isolated from human term placentas with Ce-trifluorocetate isopicnic centrifugation according to the
manufacturer’s protocol (Pharmacia, Piscataway, NJ). Poly(A)^+ mRNA was isolated with oligo(dT) cellulose (Life Technologies) as per manufacturer’s recommendations. Then 20 μg of RNA was size-fractionated and probed by sequential hybridization with the IL-1R type I and type II isoform probes generated by reverse transcription-polymerase chain reaction (RT-PCR) (see next section). The transcriptions were labeled with [α-^32P]deoxy cytidine 5'-triphosphate by random priming.

**RT-PCR and Restriction Analysis.** RT-PCR was performed with mRNA purified from JAR cells and placenta to detect the expression of IL-1 type I and type II receptors by using the published cDNA sequences as the basis for the primer design (Sims et al., 1988; McManah et al., 1991). The upstream and downstream primers specific for the type I receptor were 5'-TGGTACCGAGGAGTGATG-3' and 5'-TAGATGAAATTGCACGCCT-3', which corresponded to nucleotide positions 853 to 873 and 1705 to 1724 of the published cDNA sequence (Sims et al., 1988), respectively. 5'-GCAAAGTGGCTGGTACG-3' and 5'-CCACACGATGTTGTTAAGG-3' corresponded to nucleotide positions 59 to 77 and 861 to 880 of the published cDNA sequence (McManah et al., 1991), were the primers specific for the type II receptor. The expected size of the RT-PCR product was 872 bp for IL-1R type I and 822 bp for IL-1R type II. The RT-PCR products were run on a 1% agarose gel, and the bands were excised and purified with QIAEX II gel extraction kit (Qiagen, Chatsworth, CA). The purified products were subsequently cloned into pGEM-T vector system (Promega). For the restriction fragment analysis of the RT-PCR products, the cloned cDNA inserts were released from the plasmid by XbaI/Xhol digestion in the case of IL-1R type I and by PstI/SacII digestion in the case of IL-1R type II. The IL-1R type I cDNA insert was analyzed by digestion with EcoRI, SacI, and XmnI.

**DNA Sequencing.** The identities of the RT-PCR products cloned into the pGEM-T vector were confirmed by sequencing the clones on both 5' and 3' ends with T7 promoter and SP6 promoter primers. This was done by the dideoxy chain termination method, with the Sequenase 2.0 kit (U.S. Biochemical Corp., Cleveland, OH).

**Preparation of Brush Border and Basal Membranes from Human Placenta.** Maternal-facing brush border membranes were prepared from normal term human placentas as previously described (Balkovetz et al., 1986; Kelley et al., 1993). Final membrane preparations were suspended in 10 mM Tris-HCl, pH 7.4, at a protein concentration of 10 mg/ml. Membranes were stored in liquid N2 until used. A portion of the placental membrane preparation just before the final concentration of 0.025 to 10 nM was added in a volume of 50 μl. The assay volume was made up to 200 μl with the addition of buffer. For the calculation of nonspecific binding, 20 nM unlabelled IL-1β was used in the binding assay. The binding was allowed to proceed at room temperature for 2 h. The binding was terminated by addition of 3 ml of ice-cold stop buffer (10 mM Tris-HCl, pH 7.4), followed by filtration of the mixture on a GF/F glass fiber filter (0.7-μm pore size), which had been presoaked in 3% BSA. The filter was washed three times with 5 ml of the ice-cold stop buffer, and the radioactivity associated with the filter was determined with a gamma counter.

**Statistical Analysis.** Data are presented as means ± S.E. Statistical significance of differences between paired samples was determined by Student’s t test. A P value of <.05 was considered significant. Scatchard analysis of the ligand binding data was used to determine the kinetic parameters Kd (dissociation constant) and Bmax (maximal binding capacity).

**Results**

**Blockade of Effect of IL-1β on SERT Expression by IL-1 Receptor Antagonist.** Our previous studies have shown that treatment of JAR human placental choriocarcinoma cells with IL-1β for 16 h leads to an increase in SERT activity measured as the imipramine-sensitive serotonin uptake and that the IL-1β effect is accompanied by an increase in the steady-state levels of SERT mRNAs (Ramamoorthy et al., 1995c). Furthermore, the IL-1β-induced increase in SERT mRNA levels is prevented if actinomycin D is included during IL-1β treatment, indicating that the effect of IL-1β on the transporter expression occurs at the transcriptional level. In the present investigation, we assessed the role of IL-1 receptor in the observed effect. Two types of receptors, type I and type II, which bind IL-1β are known, of which only the type I receptor is capable of signal transduction (Dinarello, 1991). IL-1 receptor antagonist is an endogenous peptide that specifically binds to IL-1 receptors without inducing signal transduction, thus effectively blocking the biological effects of IL-1β (Arend, 1993). Therefore, to establish the involvement of IL-1 receptors in the IL-1β-induced up-regulation of SERT gene expression, we evaluated the ability of IL-1 receptor antagonist to block the effect of IL-1β (Table 1). IL-1β was found to increase SERT activity by 88%. This effect was, however, completely blocked by IL-1 receptor antagonist. Treatment of the cells with the antagonist alone did not have any effect on the transporter activity. Northern blot analysis revealed that IL-1β increased the steady-state levels of SERT mRNAs severalfold. Cotreatment of the cells with IL-1β and

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<td>IL-1β</td>
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<td>IL-1β plus IL-1 receptor antagonist</td>
<td>0.81 ± 0.03*</td>
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* P < .05 compared with other three values; ** not statistically significant from the group with no treatment; " not statistically significant from the group treated with the antagonist alone.

**Preparation of JAR Cell Plasma Membranes.** JAR cells were cultured in 225-cm^2^ culture flasks, with RPMI-1640 supplemented with 10% fetal bovine serum and penicillin (100 U/ml)-streptomycin (100 μg/ml). Plasma membranes were prepared as described in Jayanthi et al. (1994). Membrane preparations were suspended in 10 mM Tris-HCl, pH 7.4, at a protein concentration of 4 mg/ml and stored in liquid N2 until used. Alkaline phosphatase was used as the marker enzyme for brush border membranes to assess the membrane enrichment, and dihydroalprenalol binding was used to assess the enrichment of basal membrane preparations.

**Preparation of Radiolabeled IL-1β.** IL-1β was radiolabeled with [125I]NaI with the iodogen reagent (Pierce, Rockford, IL). Free label was separated from the incorporated radioactivity with BioGel P-10 gel filtration chromatography. Purified [125I]IL-1β was stored in 10 mM Tris-HCl, pH 7.4, containing 0.2% (w/v) BSA. 125I-IL-1β Binding Assay. All assays were performed in siliconized glass tubes and in a total volume of 200 μl. Forty microliters of membrane preparations (10 mg/ml protein) was diluted with 60 μl of 10 mM Tris-HCl buffer (pH 7.4) containing 0.2% BSA. 125I-IL-1β at a final concentration of 0.025 to 10 nM was added in a volume of 50 μl. The assay volume was made up to 200 μl with the addition of buffer. For the calculation of nonspecific binding, 20 nM unlabelled IL-1β was used in the binding assay. The binding was allowed to proceed at room temperature for 2 h. The binding was terminated by addition of 3 ml of ice-cold stop buffer (10 mM Tris-HCl, pH 7.4), followed by filtration of the mixture on a GF/F glass fiber filter (0.7-μm pore size), which had been presoaked in 3% BSA. The filter was washed three times with 5 ml of the ice-cold stop buffer, and the radioactivity associated with the filter was determined with a gamma counter.

**Statistical Analysis.** Data are presented as means ± S.E. Statistical significance of differences between paired samples was determined by Student’s t test. A P value of <.05 was considered significant. Scatchard analysis of the ligand binding data was used to determine the kinetic parameters Kd (dissociation constant) and Bmax (maximal binding capacity).
IL-1 receptor antagonist abolished the IL-1β effect completely (Fig. 1). Again, the IL-1 receptor antagonist by itself had no noticeable effect on SERT mRNA levels. These data clearly establish the involvement of the type I IL-1 receptor in the influence of IL-1β on SERT gene expression in JAR cells.

**Signaling Events Associated with IL-1β Treatment.** We have shown previously that the effect of IL-1β on SERT expression in JAR cells does not involve cAMP, cGMP, and nitric oxide (Ramamoorthy et al., 1995c). In the present study, we investigated the possible involvement of tyrosine phosphorylation in the effect of IL-1β. A role for tyrosine phosphorylation in the biological effects of IL-1β has been demonstrated in other cell systems (Corbett et al., 1993). We used genistein, an inhibitor of tyrosine kinases, to assess the role of tyrosine phosphorylation in the present study. It was found that the ability of IL-1β to increase SERT activity in JAR cells was abolished by cotreatment with genistein (Table 2). Thus, tyrosine phosphorylation plays a key role in the enhancement of SERT expression by IL-1β.

There is evidence from other biological systems that IL-1 is able to activate the MAP kinase cascade (Raingeaud et al., 1995; Scherer et al., 1997). Activation of MAP kinases (also called ERKs) occurs by phosphorylation of threonyl and tyrosyl residues by MAP kinase kinases (Seger and Krebs, 1995). Inhibitors of tyrosine kinases such as genistein are known to block the phosphorylation and consequent activation of MAP kinases in intact cells (Waddell et al., 1995). To examine the possibility that MAP kinase activation is one of the early events in the IL-1β-induced activation of SERT gene expression in JAR cells, we performed the kinase assay of the ERK2 antibody immunoprecipitate after treatment of the cells with IL-1β for different time periods (Fig. 2). The ERK2 antibody immunoprecipitates ERK2 p42 and, to a lesser extent, ERK1 p44. This assay measures the ability of the immunoprecipitate to phosphorylate the exogenous protein substrate basic myelin protein. Because only activated MAP kinases possess the ability to phosphorylate, this procedure detects activation of MAP kinases. The immunoprecipitate from untreated control cells showed very little phosphorylation activity. In contrast, the immunoprecipitate from IL-1β-treated cells showed enhanced phosphorylation activity. The IL-1β-dependent MAP kinase activation was evident within 5 min of IL-1β treatment. The activation was maximal at 15 min, but returned to control levels within 60 min. In two separate experiments done with two different lots of commercially available basic myelin protein, the stimulation of phosphorylation induced by 15-min treatment with IL-1β was 9- and 12-fold compared with control with no IL-1β, and 4) 10 ng/ml IL-1β plus 75 μM genistein, and 4) 10 ng/ml IL-1β plus 75 μM genistein. Following the treatment, the medium was removed and the cells were washed with the transport buffer. Imipramine-sensitive transport of serotonin (50 nM) was then measured in these cells with a 3-min incubation.

**TABLE 2** Blockade of IL-1β-induced SERT expression by genistein

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* P < .05 compared with other three values; ** P < .01 compared with the groups without genistein treatment; † not statistically significant from the group treated with genistein alone.

The primary outcome of IL-1 receptor signaling involving

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**Fig. 1.** Influence of IL-1 receptor antagonist on IL-1β-induced increase in steady-state levels of the SERT mRNAs. JAR cells were treated for 16 h at 37°C as follows: no addition (lane 1), IL-1β (lane 2), IL-1 receptor antagonist (lane 3), and IL-1β plus IL-1 receptor antagonist (lane 4). Concentrations of IL-1β and IL-1 receptor antagonist were 10 and 50 ng/ml, respectively. Cells were treated for 15 min with the antagonist before the addition of IL-1β. Poly(A)+ RNA was isolated from these cells and analyzed by Northern blot hybridization for steady-state levels of SERT mRNAs with the SERT cDNA as the probe. The same blot was then probed with GAPDH cDNA for steady-state levels of the GAPDH mRNA to serve as the internal control for RNA loading and transfer efficiency.

**Fig. 2.** Influence of IL-1β on MAP kinase activity in JAR cells. Cells were treated with 10 ng/ml IL-1β for 5, 15, 30, and 60 min. Cells that were not treated with IL-1β served as the control. Cell extracts were prepared from the cells and MAP kinases were immunoprecipitated with anti-ERK2 antibody. The kinase activity of the immunoprecipitates was then determined by assessing the ability of the precipitates to phosphorylate basic myelin protein (BMP) with [γ-32P]ATP. The proteins in the reaction mixture were separated on SDS-PAGE and phosphorylated basic myelin protein was identified by autoradiography.
the MAP kinase cascade is the activation of NF-κB (Osborn et al., 1989; Wesche et al., 1997). A majority of the target genes whose expression is influenced by IL-1 contain NF-κB-responsive elements (O’Neill, 1995). The human SERT gene has been cloned and characterized (Lesch et al., 1994). We examined the promoter region of the gene for the presence of NF-κB binding sites and found two binding sites. Therefore, it seemed possible that the IL-1 signaling in JAR cells involves the activation of NF-κB, with the activation of the MAP kinase cascade as an upstream event of the pathway. To examine this possibility, we performed EMSA for the detection of NF-κB activation with the nuclear extract from JAR cells that were treated with IL-1β for different time periods. There are several forms of NF-κB and these factors are normally retained in the cytoplasm bound by inhibitory proteins belonging to the IκB family. During the activation process in response to an external signal such as IL-1, IκB is phosphorylated and degraded, leaving the NF-κB free to be translocated into the nucleus (Baeuerle and Henkel, 1994; Baldwin, 1996). The presence of NF-κB, thus activated to function as the trans-acting factor in the nucleus, can be detected with the nuclear extract from activated cells by EMSA with 32P-labeled oligonucleotide containing the consensus sequence for the binding of NF-κB. The results of the EMSA are given in Fig. 3. The shift in the electrophoretic mobility of the nucleotide was not detectable in control cells, showing that there is no constitutive activation of NF-κB in these cells. Treatment of the cells with IL-1β however led to the shift in the electrophoretic mobility of the nucleotide. The shift was transient. It was detectable within 15 min of treatment, was maximal at 30 min and almost disappeared at 60 min. The observed shift was specific because the shift detected at 30 min could be completely blocked by the inclusion of excess unlabeled oligonucleotide in the assay.

NF-κB consists of homo- and heterodimeric proteins that belong to the Rel family of trans-acting factors. In mammals, the Rel family members include five proteins, p50, p52, p65 (RelA), c-Rel, and RelB (Baueuerle and Henkel, 1994; Baldwin, 1996). To elucidate the subunit composition of the NF-κB that is activated in JAR choriocarcinoma cells on treatment with IL-1β, we performed EMSA in the presence of antibodies against the members of the Rel protein family (supershift assay) (Fig. 4). We used a 30-min treatment with IL-1β at which time the IL-1β-induced activation of NF-κB was found to be maximal. Among the four antibodies tested (anti-p50, anti-p52, anti-p65, and anti-c-Rel), only the anti-p65 antibodies were able to retard the mobility of the NF-κB-nucleotide complex. This shows that the NF-κB that is activated by IL-1β in JAR cells contains the p65 subunit. Because no other antibody was positive in the supershift assay, the data suggest that the IL-1β-responsive NF-κB in these cells is a p65 homodimer.

**Expression of IL-1 Receptors in JAR Cells and in Normal Human Placenta.** The activation of the SERT gene in JAR cells and the effective blockade of this activation by IL-1 receptor antagonist prompted us to examine the expression of IL-1 receptors in these cells. We also examined the expression of these receptors in normal human placenta because we are not aware of any published report on IL-1 receptors in this tissue. As JAR cells are being used as a model system for the placental syncytiotrophoblast, it is important to investigate the expression of IL-1 receptors in the normal placenta to understand the physiological relevance of the results obtained with the JAR cell model system. First, we performed Northern blot analysis to see if the IL-1 receptor subtype-specific transcripts can be detected in JAR cells and in normal placenta. We used IL-1R type I and IL-1R type II cDNA probes that were generated by RT-PCR (see below). As can be seen in Fig. 5, type I IL-1 receptor mRNA (5.0 kilobases) is present in JAR cells as well as in normal placenta. It also appears that the transcript levels are more abundant in the placental tissue than in JAR cells. Northern blot analysis also provided evidence for the presence of transcripts specific for type II IL-1 receptor (1.7 kilobases). From the relative intensities of the hybridization signals, it appears that the normal placenta expresses more type I receptor than type II receptor. The transcripts for type II receptor were not detectable in JAR cells in Northern blot analysis.

To further evaluate if the IL-1 receptors expressed in JAR cells and placenta are the same and also to establish the identity of these receptors unequivocally, we performed RT-PCR and restriction site analysis of the resulting products. RT-PCR was done with primers specific for the human type I and type II IL-1 receptors. Poly(A)+ mRNA from JAR cells and from normal placenta yielded RT-PCR products of expected size for both type I (872 bp) and type II (822 bp) IL-1.
receptors (Fig. 6A), indicating that both forms of the receptors are expressed in normal placenta as well as in JAR cells. The resultant RT-PCR products were subcloned into pGEM-T vector and restriction site analysis of the cDNA inserts was then performed. In the case of type I receptor, three enzymes were used: DdeI, HindIII, and TaqI. Based on the published nucleotide sequence of human type I IL-1 receptor cDNA, the expected sizes of the fragments resulting from the digestion of the cDNA insert by these three enzymes are as follows: DdeI, 465 bp and 407 bp; HindIII, 509 bp and 363 bp; TaqI, 734 bp and 138 bp. As can be seen in Fig. 6B, the RT-PCR products from JAR cells and normal placenta yielded restriction fragments of expected size. Similar analysis was done with type II IL-1 receptor cDNA products using EcoRI, SacI, and XmnI (Fig. 6C). Again, the RT-PCR products from JAR cells and normal placenta yielded restriction fragments of expected size based on the published nucleotide sequence of human type II IL-1 receptor cDNA (EcoRI, 472 and 350 bp; SacI, 451 and 371 bp; XmnI, 483 and 339 bp). The RT-PCR products were subsequently sequenced and their identities established unequivocally. These results confirmed that JAR cells and normal placenta express both type I and type II IL-1 receptors.

**Fig. 4.** Supershift assay for determination of the subunit composition of the IL-1β-responsive NF-κB in JAR cells. Cells were treated with or without IL-1β (10 ng/ml) for 30 min. Nuclear extracts were prepared from the cells and used for the supershift assay. The assay was performed with 32P-oligonucleotide bearing the consensus sequence for NF-κB binding site in the presence or absence of 1.5 μg of anti-p50, anti-p65, anti-c-Rel, or anti-p52. Lane 1 contained 32P-oligonucleotide in the absence of nuclear extract. The specificity of EMSA was determined by including an excess of unlabeled oligonucleotide during the EMSA reaction with nuclear extracts prepared from cells treated with IL-1β for 30 min (lane 4).

**Fig. 5.** Northern blot analysis of poly(A)+ RNA from JAR cells and human normal term placenta for expression of type I and type II IL-receptors. Poly(A)+ RNA (20 μg/lane) was size-fractionated and hybridized to 32P-labeled cDNA probes specific for human type I IL-1 receptor (A) or for human type II IL-1 receptor (B). The cDNA probes were obtained by RT-PCR (see Fig. 6).

**Fig. 6.** Restriction enzyme analysis of RT-PCR products from JAR cells and normal placenta (A) or purified brush border membranes (B). Lane 1 contained 32P-oligonucleotide in the absence of nuclear extract. The specificity of EMSA was determined by including an excess of unlabeled oligonucleotide during the EMSA reaction with nuclear extracts prepared from cells treated with IL-1β for 30 min (lane 4).

**Binding of IL-1β to Membranes from JAR Cells and Normal Placenta and Polarized Distribution of the Binding Activity in Placental Syncytiotrophoblast.**

The presence of IL-1 receptors in membrane preparations can be monitored by measuring the binding of 125I-IL-1β. This approach however cannot differentiate between type I and type II IL-1 receptors because both types bind IL-1. The syncytiotrophoblast of the human placenta is the functional unit of the tissue. This cell is polarized, with its brush border (apical) membrane in direct contact with maternal blood and its basal membrane facing the fetal circulation. In contrast, the JAR choriocarcinoma cells do not polarize (Mitchell et al., 1995). The distribution of the IL-1 receptors in the brush border membrane versus the basal membrane of the placental syncytiotrophoblast is an important issue because it determines whether IL-1 in the maternal circulation or IL-1 in the fetal circulation modulates SERT gene expression in the syncytiotrophoblast. The maternal-facing brush border membranes and the fetal-facing basal membranes can be differentially isolated from crude membranes of the placenta (Balkovetz et al., 1986; Kelley et al., 1993). We measured the specific binding (i.e., the binding of 125I-IL-1β that is inhibitable by 20 nM unlabeled IL-1β) of 125I-IL-1β in crude membranes and in purified brush border membranes (Fig. 7A). The specific binding was found to be enriched in the brush border membranes ~4-fold compared with the crude membranes. This enrichment in IL-1β binding was comparable to the enrichment of alkaline phosphatase, a marker enzyme for the brush border membrane (Fig. 7B). In addition, the specific binding of IL-1β in brush border membranes is ~4-fold compared with the specific binding of IL-1β observed in basal membranes (Fig. 7C). These data show that the IL-1β binding activity is present predominantly in the maternal-facing brush border membrane of the syncytiotrophoblast.

We also measured the binding of 125I-IL-1β to plasma membranes prepared from JAR cells. Specific binding was detectable in these membrane preparations. However, the
specific activity of the binding in JAR cell membranes was 
50% compared with the binding observed in placental 
brush border membranes (8.9 ± 1.6 versus 19.4 ± 2.9 
fmol/mg protein, respectively at 2 nM ¹²⁵I-IL-1β). We carried 
out the kinetic analysis of the binding with placental brush 
border membranes. The binding of ¹²⁵I-IL-1β to the mem-
branes was inhibitable by unlabeled IL-1β in a dose-depen-
dent manner (Fig. 8A). Scatchard analysis of the specific 
binding yielded a value of 2.6 ± 0.3 nM for K_D and a value of 
66 ± 7 fmol/mg protein for B_max.

Discussion

We have demonstrated in this article that treatment of 
JAR cells (a human placental choriocarcinoma cell line) with 
IL-1β up-regulates SERT gene expression, an effect that is 
completely blockable by IL-1 receptor antagonist. We also 
have shown that tyrosine phosphorylation is involved in the 
IL-1β effect because genistein, a tyrosine kinase inhibitor, is 
able to abolish the IL-1β effect on SERT activity. Our previ-
ous studies have shown that IL-1β and tyrosine kinase in-
hbitors alter only the maximal velocity of the serotonin 
transport process without having any significant effect on the
of membrane protein) were incubated with 0.025 to 10 nM 125I-IL-1 for 2 h at room temperature. Nonspecific binding, measured in the presence or absence of increasing concentrations (0.01–10 nM) of unlabeled IL-1, was subtracted from total binding to determine specific binding. Data for specific binding were used for Scatchard analysis.

Fig. 8. Kinetics of IL-1β binding to human placental brush border membranes. A, dose-response relationship for the inhibition of 125I-IL-1β binding by unlabeled IL-1β. Purified placental brush border membranes (400 μg of membrane protein) were incubated with 0.5 nM 125I-IL-1β for 2 h at room temperature in the presence or absence of increasing concentrations (0.01–10 nM) of unlabeled IL-1β. B, Scatchard analysis of 125I-IL-1β binding to placental brush border membranes. Membranes (400 μg of membrane protein) were incubated with 0.025 to 10 nM 125I-IL-1β for 2 h at room temperature. Nonspecific binding, measured in the presence of 20 nM unlabeled IL-1β, was subtracted from total binding to determine specific binding. Data for specific binding were used for Scatchard analysis.

Substrate affinity (Ramamoorthy et al., 1995c; Prasad et al., 1997). Studies of the IL-1β-induced signaling mechanisms in JAR cells have revealed that IL-1β treatment leads to the activation of the MAP kinase cascade and to the activation of NF-κB. Because tyrosine phosphorylation is known to participate in the activation of the MAP kinase cascade in several biological systems, the ability of genistein to block the IL-1β effect most likely involves inhibition of MAP kinase kinases or other tyrosine kinases in the pathway upstream of the activation of the MAP kinases. The time course of the maximal effect of IL-1β on the activation of the MAP kinases and on the activation of NF-κB suggests that NF-κB activation follows MAP kinase activation. The maximal effect on MAP kinase is seen at ~15 min of IL-1β treatment, whereas the maximal effect on NF-κB is seen at ~30 min of IL-1β treatment. The influence of IL-1β on the activation of MAP kinase as well as of NF-κB is transient. The increase in the functional activity of SERT in response to IL-1β treatment is not seen until after 4 h (Ramamoorthy et al., 1995c), suggesting that the activation of SERT gene transcription by IL-1β follows NF-κB activation. These data show that the influence of IL-1β on SERT gene expression is associated with tyrosine phosphorylation, MAP kinase activation, and NF-κB activation.

An analysis of the published sequence of the promoter region of the human SERT gene does indicate the presence of two NF-κB binding sites, suggesting that the gene is a likely target for NF-κB.

The present study also has led to the identification of the biochemical nature of NF-κB in JAR cells. The NF-κB that is activated by IL-1β in these cells is a p65 homodimer. This is an interesting finding because the primary form of NF-κB in mammalian cells consists of a heterodimer of p50 and p65. The observation that the IL-1β-responsive NF-κB in JAR cells is a p65 homodimer is important in the light of recent studies focusing on the mechanisms of the activation of different forms of NF-κB (Simeonidis et al., 1997; Whiteside et al., 1997). NF-κB is normally located in the cytoplasm in an inactive form as a complex with the inhibitory proteins IκB. The IκB family consists of at least three members, IκBa, IκBβ, and IκBe (Baueuerle and Henkel, 1994; Baldwin, 1996). IκBa and IκBβ bind preferentially to the heterodimer forms of NF-κB containing either p50 or p52, whereas IκBe binds exclusively to p65 homodimer or p65/c-Rel heterodimer (Simeonidis et al., 1997; Whiteside et al., 1997). Because the IL-1β-responsive NF-κB in JAR cells is the p65 homodimer and contain neither p50 nor p52, it is IκBe and not IκBa or IκBβ is likely to be associated with IL-1β signaling in these cells. IL-1, in general, leads to phosphorylation and subsequent degradation of both IκBa and IκBβ and IL-1-induced NF-κB activation persists for several hours in spite of the enhanced synthesis of IκBa (Thompson et al., 1995).

Unlike most studies in which the IL-1β-induced activation of NF-κB has been found to be persistent, the activation of NF-κB by IL-1β in JAR cells is transient. The exclusive participation of IκBe in the IL-1β-induced activation of the p65 homodimeric form of NF-κB in JAR cells might be responsible, by a hitherto unrecognized mechanism, for the transient nature of NF-κB activation in these cells.

In these studies, we used the JAR cells as a model system to investigate the signaling events mediated by IL-1β. It is likely that similar signaling mechanisms participate in the biological actions of IL-1β in normal placental trophoblast cells. Normal placenta as well as JAR cells express type I and type II IL-1β receptors. The levels of mRNA for both types of the receptors are relatively much higher in normal placenta than in JAR cells. It is only the type I receptor that is involved in IL-1β signaling. The type II receptor binds IL-1β but does not transduce transmembrane signaling. It is likely that the physiological role of the type II receptor is to regulate the concentration of IL-1β available for interaction with the type I receptor. Note however that JAR cells do not polarize, whereas the normal syncytiotrophoblast is a polarized cell. Therefore, the present findings that the IL-1β binding activity exhibits a polarized distribution in the two poles of the syncytiotrophoblast plasma membrane are important. The preferential location of IL-1 receptors in the maternal-facing brush border membrane would suggest that the expression of the SERT gene in the syncytiotrophoblast is influenced by the circulating levels of IL-1 in the maternal
blood. This has significance to the function of the placenta under physiological as well as pathological conditions. Human uterine myometrium produces IL-1 and serotonin is an inducer of this process via 5-hydroxytryptamine2 serotonin receptor (Wilcox et al., 1994). Because the brush border membrane of the syncytiotrophoblast is apposed to the uterine wall separated only by the intervillous space, a functional cross talk between the placenta and the uterus may exist in vivo as depicted in Fig. 9. SERT in the syncytiotrophoblast is expressed in the brush border membrane and is thus likely to control the levels of serotonin in the intervillous space. If the serotonin levels are increased in the intervillous space, this might be expected to increase the generation of IL-1 by the uterus, which would induce the expression of the SERT gene in the syncytiotrophoblast, consequently enhancing the clearance of serotonin from the intervillous space. Because serotonin is a potent vasoconstrictor, such an efficient feed-forward regulatory mechanism may be essential to maintain the levels of this vasoactive monoamine at very low levels in the intervillous space to ensure optimal blood circulation in the uteroplacental unit. Maternal use of cocaine and amphetamines is likely to interfere with this process because the serotonin transporter is directly blocked by these drugs, leading to elevated levels of serotonin in the intervillous space.

Although speculative at this time, the findings that IL-1 is a potent inducer of SERT gene expression in the placenta also may have relevance to the function of the serotonergic neurons. IL-1 receptors are expressed in serotonergic neurons (Cunningham and De Souza, 1993; Ericsson et al., 1995; Gayle et al., 1997), implying a functional role for IL-1 in the regulation of serotonergic activity. Because the levels of proinflammatory cytokines, including IL-1, are increased in the brain during bacterial and viral infection of microglia, the expression of SERT in serotonergic neurons is likely to be increased under these pathological conditions. Such an increase in the transporter activity would result in an impairment of serotonergic neurotransmission due to decreased levels of serotonin in the synapse caused by the increased reuptake via the transporter. This might have relevance to the neurological consequences encountered in pathological conditions such as AIDS dementia complex and other infections of the central nervous system.

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