

Differential Expression of Pentraxin 3 in Fibroblasts from Patients with Major Depression

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In this study, differential display technology was used to compare gene expression in cultured fibroblasts from patients with major depression, melancholic subtype vs nonmelancholic depressives and normal volunteer controls. Genes differentially expressed in depressives and normals included an overexpressed 269 bp sequence tag showing ~95% identity with the *Homo sapiens* long pentraxin 3 (PTX3) gene sequence in the 3' noncoding region. The 269 bp complementary DNA probe hybridized with the 1.9 kb PTX3 mRNA. The densitometric analysis of slot blots showed that the mean steady-state mRNA level of PTX3 was 3.5-fold higher in the melancholic group as compared to that in normal controls and in nonmelancholic depressives ($n = 8$, all groups). Incubation experiments were then conducted: confluent fibroblasts from melancholics and normal volunteers were incubated with isoproterenol 1 μ M, dexamethasone (DEX) 500 nM, and interleukin 1 β (IL-1 β) 50 ng/ml, for 0, 0.5, 1, 4, and 24 h. mRNA was isolated and quantitated using Northern blot analysis. Isoproterenol produced no significant change in PTX3 expression; DEX produced a significant increase at 4 and 24 h; IL-1 β induced an increase in both the groups that peaked at 4 h, declining to near basal levels at 24 h. There were no differences between melancholics or controls in mean change or maximum response with either DEX or IL-1 β . The results are consistent with the reported effects of IL-1 β on PTX3 expression in mouse brain. The results are of potential importance because PTX3 is a member of the long pentraxin subfamily of acute-phase proteins, which is inducible by IL-1 β , and may play roles in neuroimmunity and neuroprotection. *Neuropsychopharmacology* (2004) 29, 126–132, advance online publication, 5 November 2003; doi:10.1038/sj.npp.1300307

Keywords: differential gene expression; human fibroblasts; major depression; pentraxin 3; cytokines; acute-phase proteins

INTRODUCTION

Due to the inaccessibility of human brain tissue, peripheral cells including lymphocytes and fibroblasts have been useful to probe inherited biochemical lesions in man (Edelstein and Breakefield, 1980). Human fibroblasts as non-neuronal tissue express genes encoding proteins involved in monoaminergic-G-protein-coupled transduction cascades similar to the expression in neuronal tissue, for example, beta adrenoreceptors, 5HT_{2A} receptors, and G-proteins. Using human fibroblasts, our group has demonstrated abnormalities in the beta adrenoceptor-cyclic AMP-mediated activation of protein kinase A (PKA) and phosphorylation of the transcription factor cyclic AMP response element-binding protein (CREB) in patients with major depression, melancholic subtype, relative to normal

volunteers and nonmelancholic depressives (Shelton *et al*, 1996, 1999; Manier *et al*, 1996, 2000). These findings are similar to the results from other investigators, including a decrease in the putative binding of cyclic AMP to PKA in post-mortem brains of patients with bipolar disorder (Rahman *et al*, 1997). Of particular relevance are the findings of Dwivedi *et al* (2000, 2002), who showed that persons who died by suicide with a history of a diagnosis of MDD (but not those without) had reduced prefrontal [³H] cyclic AMP binding (reduced B_{\max} but normal K_D) and lower PKA activity vs normal controls.

Altered gene expression has been hypothesized to be a factor in the vulnerability to mood disorders (Rossby *et al*, 2000; Duman *et al*, 2000). Given the fact that phosphorylated CREB (CREB-P) regulates the expression of genes containing CRE elements, we have hypothesized that fibroblasts with decreased PKA-CREB-P activity from depressed patients would show alterations in gene expression relative to those from controls. Accordingly, we have used differential display (Liang and Pardee, 1992) to contrast gene expression patterns in cultured fibroblasts from patients with major depression with melancholia vs normal volunteers and nonmelancholic depressives. The samples from patients with melancholia had previously

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Received 05 December 2002; revised 14 July 2003; accepted 08 August 2003

Online publication: 20 August 2003 at <http://www.acnp.org/citations/Npp0820020448/default.pdf>

been shown to have reduced PKA activity and CREB phosphorylation; the nonmelancholics and normal volunteers all had PKA-CREB-P within the normal range previously reported (Shelton *et al*, 1999; Manier *et al*, 2000). A strength of differential display is that it is capable of detecting both known and novel genes and does not require an *a priori* hypothesis.

This report describes the differential expression of the *Homo sapiens* pentraxin 3 (PTX3) gene (Breviario *et al*, 1992) in these samples, as well as results of incubations with isoproterenol, dexamethasone (DEX), and interleukin 1 β (IL-1 β). PTX3 belongs to the subfamily of long pentraxins with a C-terminal 203 amino-acid sequence comparable to the short pentraxins (eg C-reactive protein) and an N-terminal 178 amino-acid unrelated portion (Breviario *et al*, 1992; Goodman *et al*, 1996; Bottazzi *et al*, 1997). The exact function of PTX3 remains unknown. The expression of PTX3 is inducible by IL-1 β and tumor necrosis factor alpha (TNF α) in the brain (Polentarutti *et al*, 2000), endothelial cells (Breviario *et al*, 1992), and fibroblasts (Lee *et al*, 1993; Lee and Masson, 1993; Diaz *et al*, 2001). This may be significant given the extensive literature relating depression and immune dysfunction (Leonard, 2000); depressed patients have been shown to have significant elevations of cerebrospinal fluid levels of IL-1 β , IL-6, and TNF- α , effects that are antagonized by antidepressants (Levine *et al*, 1999; Castanon *et al*, 2002). Although the exact function of PTX3 remains unknown, it has been shown to associate with apoptotic cells. Ravizza *et al* (2001) have shown that PTX3 immunoreactivity is induced in the rat forebrain by seizure activity. However, expression beyond 18 h is only enhanced in areas of neurodegeneration. The authors suggest that this may be a result of a neuroprotective role for PTX3 in brain immune and inflammatory response. These results indicate that PTX3 may have an important role in mediating central and peripheral immunoreactivity.

PATIENTS, MATERIALS, AND METHODS

Collection and Culturing of Human Fibroblasts

All procedures involving human subjects were conducted after written informed consent was obtained. All procedures were conducted according to the Declaration of Helsinki of 1975. Strict confidentiality was maintained for all participants. The methods used for this project were similar to those previously described (Shelton *et al*, 1996; Liang *et al*, 2001). In brief, skin biopsies for fibroblast cultures were obtained from persons with major depression, melancholic subtype ($n=8$; seven females), major depression not meeting the diagnostic criteria for melancholia ($n=8$, seven females), and normal volunteers ($n=8$; six females); mean age 44.8 (SD=13.6), 35.7 (SD=12.1), and 37.7 (SD=12.3) (ANOVA=NS), respectively. Subjects were evaluated using the Structured Clinical Interview for DSM-IV (First *et al*, 1996). A second diagnostic interview was conducted by a senior clinician; diagnostic assignment, including subtyping, was made based on a consensus between the two interviewers. All the patients all had a principal diagnosis of major depression; secondary diagnoses were allowed. However, no patient had bipolar disorder or a current or past history of psychotic disorder.

Normal volunteer controls were free of Axis I mental disorder by DSM-IV (American Psychiatric Association, 1994). Skin samples were taken via punch biopsy; fibroblast cultures were grown in Dulbecco's modified Eagle medium (Life Technologies, Grand Island, NY) containing 10% fetal bovine serum (Hyclone, Logan, UT) at 37°C, in a humidified atmosphere containing 5% CO₂ until confluent (approximately 3 weeks), as previously described (Manier *et al*, 1996, 2000).

RNA Isolation, Reverse Transcription, and Differential Display

Confluent fibroblasts from cultures were lysed with TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA). RNA was isolated and purified as previously described (Liang *et al*, 2001). Three reverse transcriptions of each RNA sample were performed. Differential display was conducted using RNAmage Kits (GenHunter Corp., Nashville, TN, USA), ultimately utilizing 80 arbitrary upstream 13mer primers, each in combination with the three one-base anchored primers (a total of 240 reactions) to display approximately 96% of the genes expressed in the fibroblasts. The bands containing apparently differentially expressed genes (cDNAs) were excised, reamplified, and subcloned via the PCR-TRAP cloning system (GenHunter Corp., Nashville, TN, USA), and sequenced in the Vanderbilt University DNA-sequencing core facility (Liang *et al*, 2001). A total of 18 genes were differentially expressed.

Riboprobe Preparation

A double-stranded DNA template containing the reamplified band, containing a T7 phage promoter at the 5' end and an SP6 phage promoter at the 3' end was produced via PCR in an amplification reaction containing 2 μ l (2 μ M) each of T7-Lgh and Sp6-Rgh (GenHunter) in 10.2 μ l nuclease-free water, 2.0 μ l 10 \times PCR buffer, 1.6 μ l α -dNTPs (250 μ M), 2.0 μ l of the PCR-TRAP plasmid containing the DNA sequence, and 0.2 μ l Amplitaq. The PCR reaction was subsequently checked by visualizing the amplified band in a 2% agarose gel.

Synthesis of a radiolabeled antisense PTX3 RNA probe (riboprobe) for Northern blot analysis was performed with a Strip-EZ RNA SP6/T7 kit (Ambion Inc., Austin, TX), using PTX3 cDNA cloned from differential display (AAGCTTT GGTCAGAGATGAATTTTA CATTGGAAGAATAACAAAA TAAGATTTGTTGTCCATTGTTTCATTGTTATTGGGTATGC ACCTTATTACAAAAAAGATGAAACATATTTATACTG CAAGGTGACTTAACAACATATAAATGTAGTTTATGTGTG ATAATCGAATGTCACGTTTTTGAGAAAGATAGTCATATAA GTTATATTGCAAAAGGGATTTGTATTAATTTAAGACTA TTTTGTAAAGCTCTACTGTAAATAAAATATTTTATAAAA AAAAAAAGCTT). The PTX cDNA was first directionally cloned into PCR-TRAP cloning vector (GenHunter Corp., Nashville, TN) and amplified using SP6 and T7 primers (GenHunter Corp., Nashville, TN) adjacent to the cloning site. The resulting PTX3 cDNA template was gel purified and used as a template. The antisense RNA synthesis was conducted in a final volume of 20 μ l containing 7 μ l nuclease-free water, 1 μ g DNA template, 2 μ l 10 \times transcription buffer, 1 μ l 10 mM ATP, 1 μ l 10 mM CTP, 1 μ l

10 mM GTP, 5 μ l [a-32P]-UTP (12.5 μ M), and 2 μ l Sp6 polymerase. The reagents were mixed thoroughly by finger-tapping, followed by brief centrifugation. The reaction was incubated for 60 min at 37°C. Template DNA was subsequently removed by the addition of 1 μ l RNase-free DNase 1 (2 U/ μ l). The reactants were mixed by finger-tapping, microfuged briefly, and incubated at 370°C for 15 min. Unincorporated ribonucleotides were removed with G-50 spin columns (Boehringer Mannheim Corp., Indianapolis, IN).

Northern Blot and Slot Blot Analysis

Fibroblast RNA (20 μ g) was resolved by electrophoresis through 2.2 M formaldehyde/1% agarose gel in MOPS buffer (20 mM MOPS [3-(N-morpholino)propanesulfonic acid], pH 7.0, 10 mM sodium acetate, 1 mM EDTA) in gel-loading buffer containing ethidium bromide (GenHunter, Nashville TN) for about 3 h at 50 V. The gel was then digitally UV-photographed along with a fluorescent ruler using an AlphaImager 2000 (Alpha InnoTech Corporation, San Leandro, CA) for marking the positions of gel wells, 18S RNA, and 28S RNA. The RNA was transferred from the gel to a nylon membrane using the Turbo blotter Rapid Downward Transfer System (Schleicher & Schuell, Keene, NH). Transferred RNA was immobilized on the nylon membrane by UV crosslinking in Stratalinker UV Crosslinker (Stratagene, La Jolla, CA). Prehybridization (2 h) and hybridization (3 h) were conducted in 30 ml of Rapid-Hyb solution at 70°C (Amersham Pharmacia Biotech, Piscataway, NJ). After prehybridization, the membrane was hybridized with 2.5×10^7 cpm riboprobe (see Riboprobe Preparation) and washed two times (15 min each time) in 150 ml $2 \times$ SSC, 0.1% SDS at room temperature, followed by washing two times (15 min each time) in 150 ml 0.1 SSC, 0.1% SDS at 70°C. The membrane was subsequently exposed overnight to a Kronex 10T Medical X-Ray film (Sterling Diagnostic Imaging, Inc., Newark, DE).

A measure of 5 μ g of total fibroblast RNA from ($n = 8$ each) melancholic depressives, nonmelancholics, and normal volunteer controls (in nuclease-free water) was combined with 8 μ l 37% formaldehyde and 12 μ l $20 \times$ SSC (Gibco BRL Life Technologies, Grand Island, NY) and heat-denatured for 15 min at 60°C. Each denatured sample was then mixed with 5 μ l ethidium bromide solution (12 μ l of stock 10 mg/ml (Gibco BRL Life Technologies, Grand Island, NY) + 88 μ l of $6 \times$ SSC) and diluted to a total volume of 400 μ l with $10 \times$ SSC. The samples were subsequently applied to a GeneScreen Plus membrane (DuPont, Boston, MA) via a slot blot apparatus (PR 600, Hoefer Scientific Instruments, San Francisco, CA) with low vacuum. Prior to loading, the membrane was soaked in nuclease-free water followed by $10 \times$ SSC, and placed on two sheets of 3MM paper prewetted with $10 \times$ SSC. After loading, the wells were washed with 400 μ l $10 \times$ SSC. The membrane was then removed from the slot blot apparatus and placed in a Stratalinker UV Crosslinker (Stratagene, La Jolla, CA) to immobilize the RNA to the membrane. After crosslinking, the membrane was air-dried for 30 min, pencil-marked as necessary to track for the relative positions of samples, and digitally UV-photographed using

AlphaImager 2000 (Alpha InnoTech Corporation, San Leandro, CA). The AlphaImager was then used to calculate the band intensities of the ethidium bromide-stained RNA molecules in each band from this digital picture of the membrane. The intensities obtained with ethidium bromide staining of the RNA molecules bound to the membrane in the different slots were used to normalize the PTX3 slot blot band intensities for slot-to-slot loading variation (Duhl *et al*, 1992). The membrane was then placed in prehybridization solution. Prehybridization (2 h) and hybridization (3 h) were conducted in 30 ml of Rapid-Hyb solution (Amersham Pharmacia Biotech, Piscataway, NJ) at 70°C. After prehybridization, the membrane was hybridized with 2.5×10^7 cpm riboprobe (see Riboprobe Preparation above) and washed two times (15 min each time) in 150 ml $2 \times$ SSC, 0.1% SDS at room temperature, followed by washing two times (15 min each time) in 150 ml 0.1 SSC, 0.1% SDS at 70°C. The membrane was subsequently exposed overnight to Kronex 10T Medical X-Ray film (Sterling Diagnostic Imaging, Inc., Newark, DE). The autoradiograph was scanned with an AlphaImager 2000 (Alpha InnoTech Corporation, San Leandro, CA) to determine band densities. Student's *t*-test was used to determine the significance.

Incubations

Since a factor contained in serum could have been responsible for the increased PTX3 expression, fibroblast cultures from melancholics and normal volunteers were exposed for 18 h to reduced serum (0.5%) prior to the incubations. Time-response curves were generated with *in situ* incubations of the following reference compounds conducted for 0, 0.5, 1, 4, and 24 h: isoproterenol 1 μ M (Sigma Chemical Co., St Louis, MO); IL-1 β 50 ng/ml (Calbiochem, La Jolla, CA); and DEX 500 nM (Sigma Chemical Co., St Louis, MO). RNA was then extracted and mRNA quantitated by Northern Blot analysis as described earlier. The concentrations of isoproterenol, DEX, and IL-1 β chosen for incubation experiments were estimated to yield maximal response, based on previous experiments conducted by our laboratory and others for each compound (Breviario *et al*, 1992; Manier *et al*, 2000; Pariante *et al*, 2001); however, the maximal response was confirmed by constructing dose-response curves.

RESULTS

One of the differentially expressed cDNA bands was excised, the cDNA subcloned and sequenced, yielding a nonambiguous 269 bp sequence tag (Figure 1). An NCBI GenBank BLAST search (<http://www.ncbi.nlm.nih.gov>) indicated that this tag is ~95% identical with the human PTX3 gene sequence (nucleotides 1548–1816) in the 3' noncoding region (Breviario *et al*, 1992). Utilizing Northern blot analysis, the riboprobe hybridized with an approximately 1.9 kb mRNA with a molecular weight matching PTX3 mRNA (1.86 kb). Since the probe hybridized with only one band, slot blots were utilized to confirm that the differences were due to differential expression and not to a PCR artifact. The mean basal PTX3 mRNA expression in the melancholic

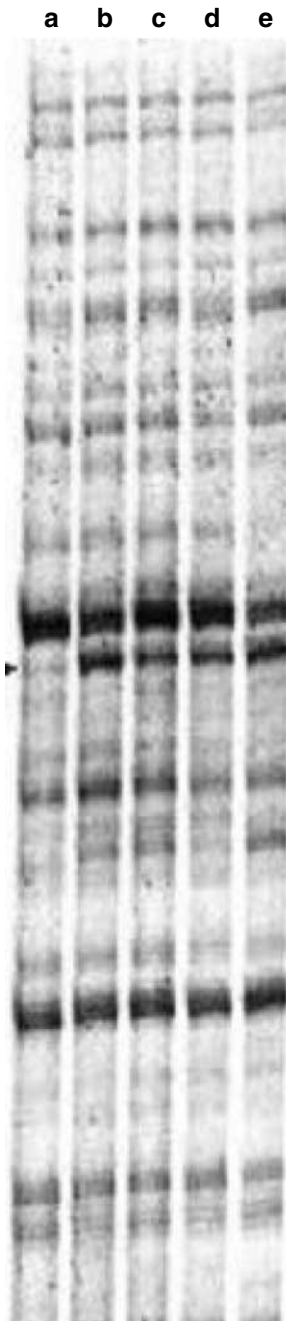


Figure 1 Autoradiogram displaying cDNA bands amplified from human fibroblast RNA utilizing HAP₂₂ as an arbitrary primer and HT₁₁C, HT₁₁G, and HT₁₁A as anchor primers. cDNAs reverse transcribed from mRNA isolated from the fibroblasts of one normal subject (a) and four patients with a diagnosis of major depression (b–e) were used as templates for amplification by PCR. The cDNA corresponding to the differentially expressed band was excised from the gel, cloned, and sequenced, as described in Patients, materials and methods.

patient group was approximately 3.5-fold higher than that in normal controls or nonmelancholics (Figure 2).

The results of the incubation experiments are depicted in Figure 3. Reduced serum decreased the expression of PTX3 in samples from both controls and melancholic depressives (mean decrease of 65% and 79%, respectively). Response to IL-1 β occurred in a time-responsive fashion, with a peak

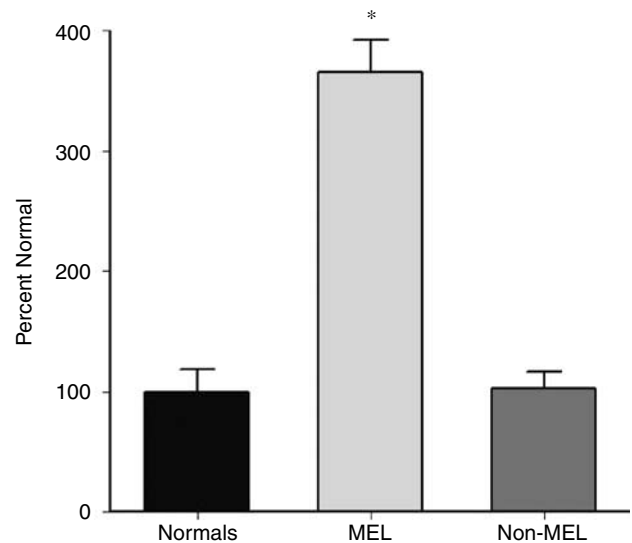


Figure 2 Densitometric analysis of PTX3 data. Riboprobe preparation and hybridization were performed as described in Patients, materials and methods. Total RNA from the fibroblasts of eight normal volunteers (black bar ■) was compared with that of eight patients with major depression, melancholic subtype (MEL) (light gray bar ■), and eight patients with major depression without melancholia (dark gray bar ■) for the expression of PTX3 mRNA. The autoradiographs were scanned with an LKB Ultrosan enhanced laser densitometer. Normalization of the band intensities for loading variation was done using the scanning data of ethidium bromide stains of the bands, as described in Patients, materials and methods. The scanning data are expressed as per cent of control values (normal subjects) \pm SEM; * $p < 0.001$.

at 4 h (mean increase, depressed = 501.1%, controls = 774.9%), declining to near basal levels at 24 h, in a fashion similar to that shown in the brain (Polentarutti *et al*, 2000). DEX produced small but significant increases at 4 h (depressed = 180.4%, controls = 220.6%), which were sustained at 24 h (depressed = 177.8%, controls = 227.1%). Isoproterenol did not produce any significant change in PTX3 at any time point. There were no differences between groups in the proportional changes in PTX3 in any of the incubation experiments.

Since the maximum response occurred for IL-1 β and DEX occurred at 4 h, a dose-response curve was generated at that time point to establish that, in fact, a maximal concentration had been used. Confluent fibroblasts were incubated with concentrations of IL-1 β of 0, 2.5, 25, 50, and 100 ng/ml; a maximum response was achieved at 50 ng/ml, yielding an EC₅₀ concentration of 1.06 ng/ml. Similarly, a dose-response curve was generated for DEX at doses of 0, 25, 250, 500, and 1000 nmol; this yielded a maximal response at 250 nmol and an EC₅₀ concentration of 4.90 nM. Isoproterenol yielded no effect in doses up to 10 μ M.

DISCUSSION

The present results indicate that there is a 3.5-fold greater basal expression of the *H. sapiens* PTX3 gene in fibroblasts cultured from patients with major depression, melancholic subtype with low PKA activity vs normal controls and nonmelancholics. It remains to be elucidated whether increased cellular production of IL-1 β or TNF- α , increased

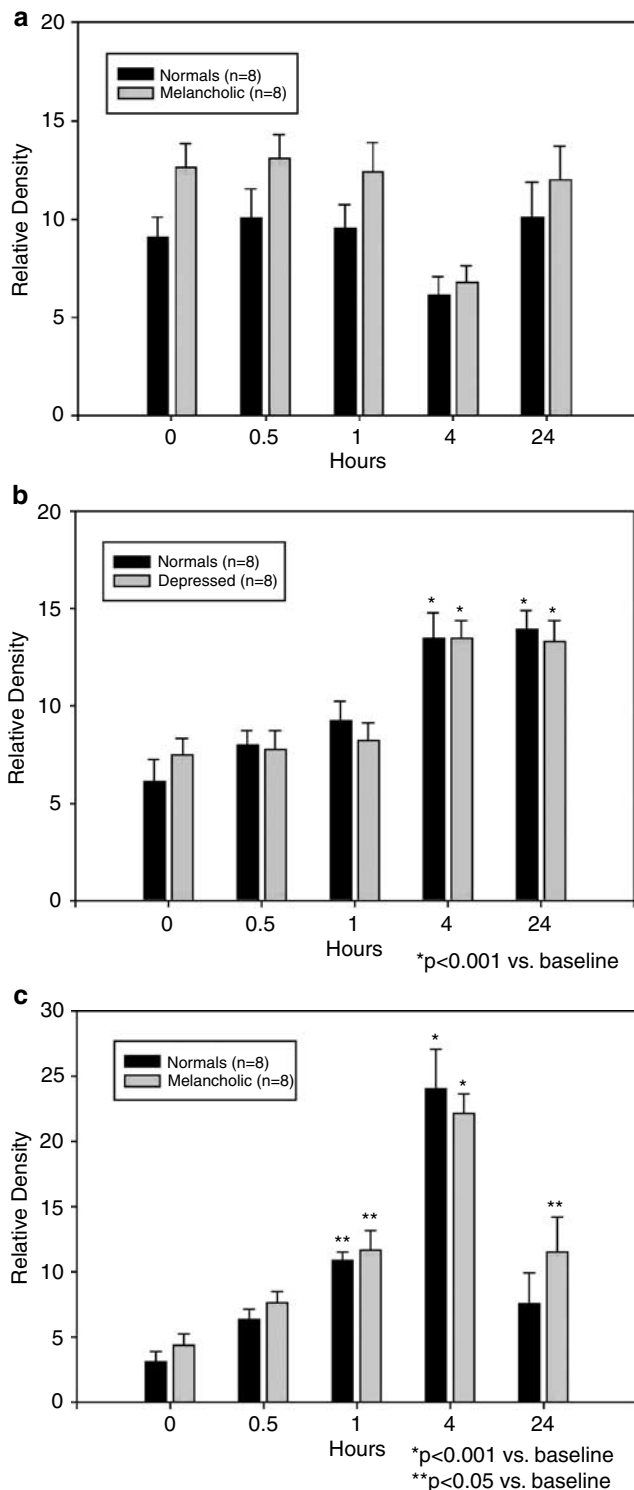


Figure 3 The effects of incubations on PTX3 expression. Confluent fibroblast cultures from normal volunteers (black bars ■) and persons with major depression, melancholic subtype (gray bars ▒) ($n = 8$ per group) were incubated for 0.5, 1, 4, and 24 h with isoproterenol $1 \mu\text{M}$ (a), DEX 500 nM (b), and IL-1 β 50 ng/ml (c), after 18 h of reduced serum exposure. PTX3 expression was determined as described in Patients, materials and methods, and compared with the basal expression in each experiment. The autoradiograph was scanned with an LKB Ultrascan enhanced laser densitometer. Normalization of the band intensities for loading variation was done using the scanning data of ethidium bromide stains of the bands, as described in Patients, materials and methods. The scanning data are expressed as normalized relative densities \pm SEM; * $p < 0.001$, ** $p < 0.05$ (vs 0 h).

basal expression of the gene itself, or alterations in another regulatory factor could explain the altered expression of PTX3 in human fibroblasts in culture.

The incubation of fibroblast cultures with isoproterenol did not produce a significant change in PTX3 expression. Incubation with isoproterenol was conducted since the cells chosen for study came from melancholic depressed patients, who had been previously shown to have reduced activity of isoproterenol-stimulated PKA activity relative to normal controls. However, no clear relationship between isoproterenol stimulation and PTX3 gene expression was shown. This suggests that the altered expression of PTX3 is not directly related to decreased PKA activity; however, factors regulated by genes linked to the PKA-CREB-CRE transduction cascade could be responsible for the effect.

Alternatively, the incubation of fibroblast cultures with IL-1 β produced a time-dependent increase in PTX3 expression over 4 h that declined to near baseline levels at 24 h. The maximal increase in expression of PTX3 at 4 h was about 500% for melancholics. The change in expression did not significantly differ between the two patient groups and controls; it is possible that significant differences could be demonstrated with a larger sample. Future experiments with an enlarged sample will need to be conducted to test whether there are significant differences between groups in maximal response to IL-1 β . Nonetheless, these data are consistent with the response to IL-1 β in CNS (Polentarutti *et al*, 2000), and support the identity of the expressed species as PTX3. However, our longer-term data suggest a decrease in the responsiveness to IL-1 β over time, suggesting a counter-regulatory response. This has not been described previously, to our knowledge. Abnormalities of cytokine activity in melancholia have been described (Anisman *et al*, 1999). However, the differences in PTX3 expression in the present experiment are not explainable by dissimilarities in plasma interleukins, since the fibroblasts from patients and controls were cultured in the same media. This would suggest that a factor intrinsic to the cells themselves would account for the findings. In addition, the incubation experiment indicated that PTX3 response to IL-1 β was downregulated at 24 h; therefore, increased cellular expression of IL-1 β seems unlikely to be the cause of differences in PTX3 expression. Consequently, factors other than IL-1 β are likely to account for the effect and need to be explored further.

DEX also produced a significant enhancement of expression of PTX3 at 4 h, which was sustained at 24 h. As far as we are aware, this is the first demonstration of an enhancement of PTX3 expression, including longer-term induction, in any tissue type by glucocorticoids (Agnello *et al*, 2000). This is consistent with the effects of glucocorticoids on other acute-phase proteins, however (Baumann *et al*, 1991). Alternatively, Agnello *et al* (2000) showed that experimental autoimmune encephalomyelitis elevated PTX3 expression and DEX administration abolished the increase in PTX3. The disparity in findings between experiments may be a result of differences in tissue type or variation in experimental conditions; in our study, DEX was administered to normal cells, while Agnello *et al* (2000) examined the effect of DEX administration on the PTX3 induction that, presumably, was a result of inflammation.

Although there was an increase in basal expression in PTX3 when cells were incubated with fetal calf serum, there was a substantial reduction in expression in the reduced-serum medium. This suggests that a serum factor could be associated with the difference in expression. The differential expression does not seem to be directly associated with either IL-1 β or DEX; although both significantly increased PTX3 expression, there was no difference seen between patients and controls. Therefore, another factor that regulates the expression of the gene may be involved. However, the demonstration of an increase in PTX3 with both IL-1 β and DEX is similar to that seen in the CNS, and supports a role for PTX3 in cellular stress response.

The findings of this study were only in fibroblasts, a peripheral cell type that may or may not show gene expression homology with brain. Furthermore, the number of participants was small; replication in a larger sample is needed. As noted earlier, the ultimate function of PTX3 is unknown, although it is associated with neuronal apoptosis and may have a neuroprotective role (Ravizza *et al*, 2001). If the increased expression of PTX3 is confirmed in the brains of patients with depression, the finding will add to a growing body of evidence, suggesting neuroendocrine-immune dysfunction in melancholia (Miller *et al*, 1999; O'Connor *et al*, 2000).

ACKNOWLEDGEMENTS

The present studies were supported by NIH grants MH-01741, MH-52339, and by a grant-in-aid from Wyeth-Ayerst Research. Dr Chakrabarti is the recipient of a NARSAD Young Investigator Award. The data were presented at the Annual Meeting of the American College of Neuropsychopharmacology, Waikaloa, HI, 2001. We thank Mrs Gloria Hopkins and Waverly Demers for the preparation and typing of the manuscript and Dr Elaine Sanders-Bush for helpful discussions and expert advice.

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