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Naltrexone influences protein kinase Cε and integrin α7 activity in SH-SY5Y neuroblastoma cells

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Abbreviations: ERK, extracellular signal-regulated kinase; GABA, gamma aminobutyrate; MEK, mitogen-activated protein kinase kinase; PI, phosphoinositide; PKC, protein kinase C; RT-PCR, reverse transcriptase-polymerase chain reaction

Abstract

Alcohol influences the neuroadaptation of brain cells where receptors and enzymes like protein kinase C (PKC) exist. Naltrexone acts on opioid receptors. However, other mechanisms of action remain unknown. We prepared SH-SY5Y neuroblastoma cells, and fed them with 150 mM ethanol for 72 h followed by treatment with naltrexone for 24 h. We performed microarray analysis and reverse transcriptase-polymerase chain reaction. Our results showed that PKCε increased 1.90 times and showed an overall decreasing pattern as time increased. Phosphorylated ERK also increased 2.0 times according to the change of PKCε. Integrin α7 increased 2.32 times and showed an increasing pattern as time increased. In conclusion, naltrexone influences PKCε neuronal signaling system and endothelial adhesion molecule integrin α7 in addition to the well-known opioid system.

Keywords: alcohols; DNA microarray; ITGA7 protein, human; naltrexone; neuroblastoma; protein kinase C

Introduction

Naltrexone is known to diminish the craving for alcohol by increasing opioid receptors. Worldwide, naltrexone became widely used after the Food and Drug Administration (FDA) approved its use as a therapeutic agent for alcoholism in 1994. However, the brain mechanism that causes alcohol addiction is not completely understood. It is known that naltrexone acts on opioid receptors, but the complete pathway of the therapeutic effect is unknown.

One strategy for developing therapies for alcoholism focuses not on receptors but on the enzymes within the cell that are involved in the brain's neuroadaptation to alcohol. Chronic drinking alters the distribution of these enzymes within the cells and can change the way brain cells respond to alcohol (Hodge *et al*., 1999; Hoffman *et al*., 2003; Nuwayhid and Werling, 2003; Proctor *et al*., 2003). An enzyme called protein kinase C (PKC) is the subject of significant research because it helps shape the level of sensitivity to alcohol's behavioral effects, at least in part, through its interaction with the γ-aminobutyrate type A (GABA-A) receptor. A study examining PKC and alcohol sensitivity showed that mutant mice lacking a form of PKCε were more sensitive than their littermates to the behavioral effects of alcohol. This suggests that medications developed to inhibit PKCε could reduce the alcohol reward as well as help treat seizures but without the sedative effects of other drugs that act on GABA neurotransmission (Li, 2004).

In general, it is believed that the cyclic adenosine 3',5'-monophosphate (cAMP) and the phosphoinositide (PI) signal-transduction pathways may be the intracellular targets that mediate the action of ethanol and ultimately contribute to the molecular events involved in the development of ethanol tolerance and dependence. PKC, which is a key step in the PI-signaling cascade, has been shown to be altered in a variety of cell systems by acute or chronic ethanol exposure (Pandey, 1998).

Research examining the neuronal signaling system and microarray analyses of ethanol dependence is actively being conducted (Worst and Vrana, 2005), but little research exists regarding naltrexone. Previously, Toll *et al* (1997) reported SH-SY5Y neuroblastoma cells, which contain opioid receptors, specifically the delta 2 opioid receptor. Further research in this field could focus specifically

the neuronal signaling system created by the reaction of naltrexone with the opioid receptor. We conducted a microarray analysis of SH-SY5Y neuroblastoma cells in order to understand the molecular events and action mechanism of naltrexone.

Materials and Methods

Cell culture and ethanol/naltrexone treatment

SH-SY5Y cells were grown in minimum essential medium/ F-12 nutrient mixtures, 10% fetal bovine serum (GibcoBRL), 100 IU/ml penicillin, and 100 μg/ml streptomycin. Cultures were maintained at 37°C in 95% air/5% CO_2 humidified atmosphere. The medium was renewed every two days.

In all experiments, cells were plated on polystyrene tissue culture dishes at a density of 1×10^6 cells/100-mm plate. After 12 h, cells were fed fresh media with 150 mM ethanol. The medium was renewed with 150 mM ethanol every day. From the third day, cells were treated with 150 mM ethanol either with 100 μM naltrexone (naltrexone-treated) or without naltrexone (control) for 6 h, 12 h, and 24 h. In this study, 10 independent cultures of naltrexone-treated cells and untreated cells from a single experiment were used.

Total RNA isolation and cDNA microarray

The total RNA in the cells was extracted using TRIzol (Invitrogen, Carlsbad, CA; Shim *et al*., 2005). Gene expression was analyzed using TwinChip™ Human-8K (Digital Genomics, Seoul, Korea) including approximately 8,000 genes. RNA from the control- or naltrexone-treated cells for 12 h was reverse transcribed to cDNA with moloney murine leukemia virus reverse transcriptase using Cy3- and Cy5-dUTP, respectively. Two cDNA arrays were prehybridized at 42°C for 45 min in pre-hybridization buffer and hybridized at 58°C for 16 h in hybridization buffer with the cDNA of control and naltrexone-treated cells. Arrays were washed at 58°C for 10 min in 2X sodium chloride sodium citrate buffer (SSC), 1% sodium dodecyl sulfate (SDS) twice and at room temperature for 10 min in 0.1X SSC/0.1%SDS and for 1 min in 0.1X SSC 4 times. After the chips dried, they were scanned using Scanarray (DSS Imagetech, New Delhi, India). GenePix software (Axon Instruments, Union City, CA) was used to analyze the DNA data. The data were normalized in an intensity-dependent manner using a scatter plot smoother 'lowess' (Yang *et al.,* 2002). The genes with significant differences in gene expression was analyzed using the Significance Analysis of Microarrays software program (SAM; Tusher *et al*., 2001; Version 1.10). The genes were cosidered differentially expressed when the logarithmic gene expression ratios in four independent hybridizations were more than a 0.5-fold difference in the expression level with 5% of *q* value cutoff.

Real-time RT-PCR

Real-time RT-PCR was performed using ABI PRISM® 7500HT sequence detection system (Applied Biosystems, Foster City, CA). The reaction mixture for SYBR® Green assay contained 2X SYBR® Green PCR Master Mix (Takara, Japan), 10 pmol of forward and reverse primers and 0.6 μg cDNA from the control- or naltrexone-treated cells for 6 h, 12 h, and 24 h (Savli *et al*., 2004). The thermocycling program was 40 cycles of 95°C for 30 s and 60°C for 30 s and 72°C 30 s with an initial cycle of 95°C for 10 min. The PKCε primer set used was: forward 5'-ACCAAGGACCGCCTCTTTTT-3' and reverse 5'-AACCGTGAACGAGGCTCGT-3'. The integrin α7 primer set used was: forward 5'-AGGTGCCCAGGTCACCTTCT-3' and reverse 5'-GCTCACTGATCGTGGCCAA-3'. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer set used was: forward 5'-TGCACCACCAACTGCTTAGC-3' and reverse 5'-GGCATGGACTGTGGTCATGAG-3'.

All amplifications and detections were carried out in a 96-well reaction plate with optical caps (Applied Biosystems, Foster City, CA). At each cycle, the accumulation of PCR products was detected by monitoring the increase in fluorescence of the reporter dye from the dsDNA-binding SYBR® Green. After PCR, a dissociation curve (melting curve) was constructed in the range of 60°C to 95°C (Lyon *et al*., 2001). All data were analyzed using the $2^{-\Delta\Delta CT}$ method (Pfaffl *et al*., 2002; Baik *et al.,* 2005). Briefly, the threshold cycle (CT) values, defined as the number of cycles it takes for a sample to reach the level where the rate of amplification is the greatest during the exponential phase, were obtained for the PKCε, integrin α7 and GAPDH genes in the control- and naltrexone-treated cells. The ΔCT value for the PKCε or the integrin α7 gene was calculated using the equation: [ΔCT (control or naltrexone) = CT (PKCε or integrin α7) − CT (GAPDH)]. The fold change in the gene expression of the PKCε or the integrin α7 was finally obtained from the formula $2^{-\Delta\Delta CT}$, where the ΔΔCT (PKCε or integrin α7) value was the difference between ΔCT (naltrexone) and ΔCT (control) values. The value of $2^{-\Delta\Delta CT}$ for the control cells, which were not treated with the control- and naltrexone-treated cells, was 1. A one-fold change indicates no change, 1 above up-regulation and 1 below, down-regulation, in the gene ex-

Table 1. Microarray chip data from microarray analysis of naltrexone sorted by known functions.

Log ratio		Gene_Symbol	GenBank_ACC	Title	Biological_Process
Apoptosis	1.01	DAP3	AA207194	Death associated protein 3	Apoptosis
	0.95	PRKCE	X65293	Protein kinase C, epsilon	Induction of apoptosis
	-0.85	OPA1	AB011139	Optic atrophy 1 (autosomal dominant)	Axon transport of mitochondrion
	-0.78	MX1	AA477235	Myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	Induction of apoptosis
Cell cycle	1.01	MRE11A	AF073362	MRE11 meiotic recombination 11 homolog A (*S. cerevisiae*)	Double-strand break repair via nonhomologous end- joining
	0.89	NOLC1	Z34289	Nucleolar and coiled-body phosphoprotein 1	Cell cycle
	0.84	AKIP	AI031800	Aurora-A kinase interacting protein	_
	0.8	PTMS	AA459196	Parathymosin	DNA replication
	-0.88	CCNG1	U53328	Cyclin G1	Cell cycle
	-0.85	PPP6C	X92972	Protein phosphatase 6, catalytic subunit	G1/S transition of mitotic cell cycle
Cell growth	0.69	CCND1	X59798	Cyclin D1 (PRAD1: parathyroid adenomatosis 1)	G1/S transition of mitotic cell cycle
	-0.78	MEIS1	U85707	Meis1, myeloid ecotropic viral integration site 1 homolog (mouse)	Cell growth and/or maintenance
Immune response	0.91	NK4	AI539055	Natural killer cell transcript 4	Cell adhesion
	0.9	CD209L	AA448002	CD209 antigen-like	Antigen presentation
	-1.11	C8B	M16973	Complement component 8, beta polypeptide	Complement activation, alternative pathway
	-0.91	CD58	Y00636	CD58 antigen, (lymphocyte function-associated antigen 3)	Antimicrobial humoral response (sensu Vertebrata)
Response to stress	1.01	MRE11A	AF073362	MRE11 meiotic recombination 11 homolog A (*S. cerevisiae*)	Double-strand break repair via nonhomologous end- joining
	0.8	PTMS	AA459196	Parathymosin	DNA replication
Signal transduction	1.16	ITGA7	NM_002206	Integrin, alpha 7	Cell-matrix adhesion
	1.07	CPE	X51405	Carboxypeptidase E	Metabolism
	0.95	PRKCE	X65293	Protein kinase C, epsilon	Induction of apoptosis
	0.9	CD209L	AA448002	CD209 antigen-like	Antigen presentation
	-1.04	CHRNB1	X14830	Cholinergic receptor, nicotinic, beta polypeptide 1 (muscle)	Cation transport
	-0.88	RTKN	AA582951	Rhotekin	Signal transduction
Transcription	0.82	POLR2D	AA582562	Polymerase (RNA) II (DNA directed) polypeptide D	Transcription
	-0.97	SP3	X68560	Sp3 transcription factor	Regulation of transcription, DNA-dependent
	-0.92	PC4	AA477295	Activated RNA polymerase II transcription cofactor 4	Regulation of transcription from Pol II promoter
	-0.9	KHSRP	U94832	KH-type splicing regulatory protein (FUSE binding protein 2)	mRNA-nucleus export

pression.

Western blot analysis

Cells were immediately lysed in a Pro-Prep™-sample buffer (iNtRON biotechnology, Korea). Protein concentration was determined by the BCA protein assay kit (Pierce Biotechnology Inc., Rockford, IL). Aliquots of crude extracts (containing 20 μg of protein) were then subjected to electrophoresis on a 12% SDS polyacrylamide gel, and proteins were electroblotted onto microporous polyvinylidene difluoride (PVDF) membranes (Schleicher & Schuell Bioscience, Inc., Keene, NH). The membranes were blocked for 1 h, washed, and incubated overnight with primary antibodies at 4°C. After washing steps, the blots were visualized with enhanced chemiluminescence (ECL™ Plus Western Blotting Detection Kit; Amersham Biosciences, Piscataway, NJ). For Western blot analysis, monoclonal antibodies raised against a peptide corresponding to phospho-MEK1/2 (Ser217/221) and phospho-ERK1/2 (Thr202/Tyr204) were used (Cell Signaling Technologies, Beverly, MA). For the control of GAPDH expression, mouse polyclonal antibody was used (Santa Cruz Biotechnology, Santa Cruz, CA).

Result and Discussion

It is well known that naltrexone acts on the opioid receptors, however, additional mechanisms remain unknown. Additionally, alcohol's mechanism of action is not fully characterized. A better understanding of this mechanism could be elucidated through experiments examining naltrexone's mechanism of action. SH-SY5Y neuroblastoma cells with the delta 2 opioid receptor are appropriate to use for research on both the mechanisms of alcohol and naltrexone.

Our research was conducted under conditions similar to the drinking habits of most alcoholic patients. The media were controlled for 72 h with 150 mM of ethanol and exchanged every 12 h. In addition, we performed RT-PCR experiments at 0, 6 h, 12 h, and 24 h and microarray analysis at 12 h.

The chip data from the microarray analysis of naltrexone was sorted according to known functions (Table 1). Upon review of the chip data and previous reported references (Ellingson, 2005; Sacanella *et al.*, 2005) about intracellular targets and molecular events of alcohol and naltrexone, we decided to conduct real-time RT-PCR experiments regarding PKCε and integrin α7 among 35 candidate genes.

It is conceivable that alcohol as well as naltrexone could have an effect on neuroblastoma cells, which are known to contain opioid receptors. However, significant results were not obtained with regard to the opioid receptors. This can be explained by the differing reactions of naltrexone in regions of the brain, such as cortical or non-cortical, as well as by the opioid receptor subtype. We used SH-SY5Y neuroblastoma cells, which contain δ2 opioid receptors, for our research. When compared to receptor studies, microarray analysis is limited in determining specific receptor action. Therefore, a significant result was not obtained.

PKCε increased 1.90 times, and we believe this is related to the neuronal signaling system of alcoholism. Integrin α7, which is known as one of the soluble endothelial adhesion molecules related to alcohol-induced hepatopathy, increased 2.32 times (Table 1). RT-PCR was conducted at 0, 6 h, 12 h,

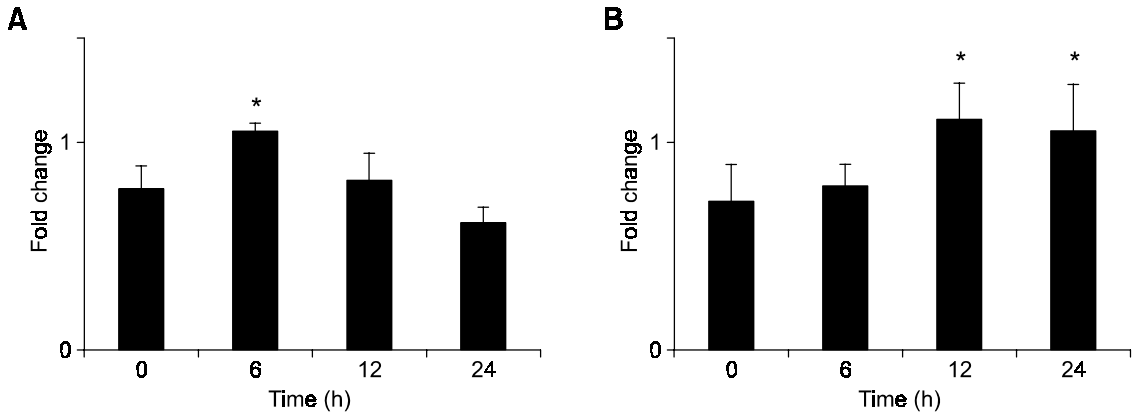

Figure 1. Real-time RT-PCR of PKCε and integrin α7 in the naltrexone-treated SH-SY5Y cells. The result of the microarray experiments was confirmed by real-time RT-PCR. The RNA isolated from 100 μM naltrexone and/or 150 mM ethanol treated SH-SY5S cells for 0 (control), 6 h, 12 h, and 24 h. Four independent experiments were performed. The fold change for the PKCε or integrin α7 was calculated as indicated in the text. For statistical analysis, we conducted a paired *t*-test. A panel; PKCε, B panel; Integrin α7; * indicates $P < 0.05$.

and 24 h for PKCε and integrin α7 which are relevant to alcohol (Figure 1).

PKCε increased 1.90 times in the microarray analysis. Results of the RT-PCR experiment at 0, 6 h, 12 h, and 24 h were; 0.78, 1.05, 0.82, and 0.61 fold, respectively. The RT-PCR results were the highest at 6 h, and tended to decrease at 12 and 24 h.

PKC, which is involved in a key step in the PI signaling cascade, represents a molecular locus for the action of ethanol and is possibly involved in the neuroadaptational mechanisms to prolonged ethanol exposure. Based on the literature, it appears that PKC plays an important role in modulating the function of various neurotransmitter receptors, including GABA-A, *N*-methyl-D-aspartate, serotonin 2A and 2C, and muscarinic receptors, resulting from ethanol exposure (Pandey, 1998). Alterations in the cAMP and the PI signaling cascades during chronic ethanol exposure could be the critical molecular events associated with the development of ethanol dependence. PKC appears to play a key role in the anxiolytic and hypnotic behavior associated with alcohol consumption. This includes the severity of the ethanol withdrawal effects, ethanol craving, possibly the predisposition to consume ethanol, the development of fetal alcohol syndrome neuropathology, the ethanol modulation of Ca^{2+} channels in the brain and heart, and the protection and cardiac damaging effects of ethanol (Ellingson, 2005).

Most studies in this area have been related to the role of PKCε as a potential target for the development of medications (Hodge *et al*., 1999; Hoffman *et al*., 2003; Nuwayhid and Werling, 2003; Proctor *et al*., 2003). The results of these studies indicate that mutant mice lacking PKCε are supersensitive to the sedative and locomotive enhancing effects of ethanol and $GABA_A$ positive modulators and consume significantly less alcohol in two-bottle choice tests. In the experiment of PKCε knockout mice using operant alcohol self-administration procedures, results indicated that PKCε knockout mice self-administered less ethanol during overnight 16 h sessions, reduced severity of alcohol-induced withdrawal seizures, which correlated with regional differences brain activity, as measured by c-fos immunohistochemistry (Olive *et al*., 2000; 2001) and showed less anxiety.

In our study, RT-PCR results were the highest at 6 h, and tended to decrease at 12 and 24 h. In the case of ethanol and naltrexone-treated SH-SY5Y neuroblastoma cells, PKCε decreased as time increased. Consequently, naltrexone acted as an antagonist on PKCε. This data suggests that PKCε is a valid target for the development of medications to treat alcohol abuse and alcoholism. Antagonist compounds could be useful in reducing craving, preventing relapse, blocking biochemical effects of alcohol, diminishing the withdrawal syndrome, and attenuating co-occurring psychiatric disorders such as anxiety.

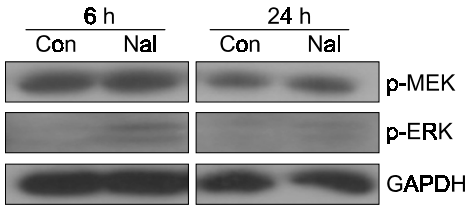

Figure 2. Western blot analysis of phosphorylated MEK1/2 and phosphorylated ERK1/2 in the naltrexone-treated SH-SY5Y cells. The cells were incubated with 100 μM naltrexone for 6 h (Nal 6 h) and 24 h (Nal 24 h). The levels of phosphorylated MEK1/2 were not changed. However, the levels of phosphorylated ERK1/2 were increased at 6 h and 24 h.

For the validation of PKC increase, we studied about the phosphorylation of extracellular signal-regulated kinase (ERK). Phosphorylated ERK also increased 2.0 times according to the change of PKCε (Figure 2). Anchoring and scaffolding homer protein regulate the membrane trafficking of mGluRIs and link mGluR5 to the ERK signaling cascade, which results in *c-fos* gene expression. Growing evidence suggests a role for the activation of mGluRIs in mediating the behavioral actions of cocaine and ethanol. This receptor family consists of mGluR1 and mGluR5, and receptor activation leads to the activation of phospholipase C (PLC) through Gαq. This, in turn, generates lipid second messengers and releases calcium from intracellular stores, which results in PKC activation. An mGluR5-specific antagonist has been shown to decrease voluntary ethanol consumption in a PKCε-dependent manner (Chiamulera *et al*., 2001).

Integrin α7 increased 2.32 times in the microarray analysis. The results of the RT-PCR experiment at 0, 6 h, 12 h, and 24 h were; 0.72, 0.79, 1.11, and 1.06, respectively. The results of the RT-PCR experiment increased as the time increased from 6 h to 12 h, but then decreased relatively at 24 h.

The integrins are heterodimeric glycoproteins composed of two chains (alpha and beta) and are essential for many leukocyte functions outside the vessel, as well as leukocyte-endothelial interactions. Up to 7 subfamilies of beta integrins have been identified on the basis of beta chain expression. It is possible that a similar situation may be observed in chronic alcoholics. In chronic alcoholics with liver disease (mainly acute alcoholic hepatitis), there is a strong correlation between serum levels of soluble endothelial adhesion molecules and clinical evolu-

tion of the severity of alcoholic hepatitis or the degree of histological injury. It is unknown whether alcoholic patients with a high ethanol intake, and thus high concentrations of soluble endothelial adhesion molecules, who, after withdrawal, become abstinent or moderate ethanol consumers (up to 40 g/day), reduce their cardiovascular risk to the same degree that the serum concentrations of endothelial adhesion molecules is reduced (Sacanella *et al*., 2005).

In the case of ethanol and naltrexone-treated SH-SY5Y neuroblastoma cells, integrin α7 increased as time increased. Consequently, naltrexone increases integrin α7 and treats histological injury.

To conclude, in the case of ethanol and naltrexone-treated SH-SY5Y neuroblastoma cells, PKCε decreased as time increased. Resonable conclusion of our research, we came to find that naltrexone may act as an antagonist on PKCε. In the case of integrin α7, the ethanol and naltrexone-treated SH-SY5Y neuroblastoma cells increased as time increased. Resonable conclusion of our research, we came to discover that naltrexone may increases integrin α7 and treat histological injury. As shown by the results above, naltrexone might act on both the neuronal signaling system and endothelial adhesion molecules beside the well-known opioid system.

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