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Alteration of lymphocyte opioid receptors in methadone maintenance subjects

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ABSTRACT

Methadone maintenance therapy is the most widely used treatment in patients with heroin addiction. Multiple studies have suggested that both current and former heroin addicts entering a methadone maintenance treatment program have altered immune function. Our previous study indicated that heroin addicts have depressed mitogen-stimulated lymphocyte proliferation and a decrease in the modulation of lymphocyte surface markers. This immunosuppression may be mediated via the direct interaction of opiates with lymphocyte opioid receptors. In order to test this hypothesis, the levels of opioid receptors on immune cells obtained from heroin users were determined using saturation binding, and it was found that former heroin addicts on methadone maintenance treatment had a significantly reduced maximum number (B_{max}) of [^{3}H]naloxone binding. The B_{max} values were 51.3 ± 7.6 fmol/mg protein for the non-addicted group and 25.3 ± 3.1 fmol/mg protein for the methadone maintenance group. Opioid receptor gene expression on the immune cell was determined using a semi-quantitative reverse-transcription polymerase chain reaction technique with specific pairs of primers to amplify mu- and delta-opioid receptor mRNAs. Both types of mRNAs were significantly decreased in lymphocytes obtained from the former heroin addicts on methadone maintenance subjects. Similarly, in an *in vitro* study, 100 μM methadone significantly down-regulated both mu- and delta-opioid receptor mRNA expressions in cultured lymphocytes obtained from naïve subjects. This effect was prevented by including 100 μM naloxone or pretreating with 50 ng/ml pertussis toxin. The data presented indicate that chronic opiate exposure was associated with down-regulation of G-protein-coupled opioid receptor gene expression in human lymphocytes.

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1. Introduction

Methadone was developed in the early 1960s as a treatment for heroin addiction (review, Kreek, 2000). Methadone maintenance therapy is the standard treatment for heroin addiction, and continues to be the most effective method of managing addiction diseases. Methadone maintenance therapy is one of the most researched, evidence-based treatments for illicit drug dependence. It is important to emphasize that studies have demonstrated that methadone maintenance therapy reverses the abnormalities in neuroendocrine and immune system function that are associated with heroin addiction (Kreek, 1992, 1997).

Despite the large number of studies that have explored the basic principles of methadone maintenance during the previous decades, the majority of health care providers have little or no understanding of either the concept of maintenance or the neurobiological actions of methadone. Multiple studies suggest that current and former heroin addicts entering the methadone maintenance program have altered immune functions (See Kreek, 1990 for review). However, since the start of the AIDS epidemic, studies dealing with immunological function of heroin addicts have become more difficult to perform and more complex to interpret. Even in the absence of AIDS, there is increasing evidence that chronic opiate use can affect the functioning of the immune system. Heroin addicts have an increased susceptibility to a variety of infectious diseases and alterations in a wide variety of immune parameters (Rouveix, 1992; Carr et al., 1996). In addition, a variety of changes in immune system function have been observed, which suggest that heroin addicts have both decreased and increased immune system function. Both the absolute numbers and the percentages of the total and the active T-lymphocytes (McDonough et al., 1980; Govitrapong et al., 1998), as well as T-cell rosette formations (McDonough et al., 1980) were significantly decreased in the peripheral blood obtained from opiate addicts. In our previous study

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(Govitrapong et al., 1998), we found that, compared to controls, T-cells from heroin addicts and subjects in 1–5-day heroin-withdrawal had decreased responses to PHA stimuli, and that morphine had an *in vitro* effect. A suppressed PHA response in methadone subjects has also been reported (Quagliata et al., 1977).

The finding that opioid receptors exist within the immune system was crucial for determining that immune cell function was directly altered by opioids. Opioid receptors expressed by immune cells are often identical to neuronal opioid receptors. Thus, depressed immune system function may be due to a direct mechanism involving opioid receptors on immune cells.

A recent study (Martin et al., 2007) indicated that methadone has a greater relative intrinsic efficacy than the active metabolites of heroin at mu-opioid receptors, and the extent of mu-opioid receptor desensitization is dependent on agonist efficacy. This finding raised questions about the possible role of exogenous opiates, especially long-term methadone, on altering immune function. The immunomodification may be mediated directly through the effect of the opiate on the opioid receptors of immune cells. Therefore, in this study, the major emphasis was on assessing opioid receptor and receptor gene expression levels on lymphocytes obtained from methadone maintenance subjects.

2. Materials and methods

2.1. Subjects

The subjects ($n = 18$) were patients attending the methadone maintenance program of Thanyarak Hospital and a control group of healthy adults ($n = 18$). The methadone maintenance subjects were previous parenteral heroin-abuse outpatients (duration of heroin-abuse, 9.8 ± 3.0 years; dosage used, 360 ± 201 mg/day) who came to the hospital daily and received methadone syrup orally. The methadone dosage was 20 mg/day, and the duration of methadone maintenance therapy was 2.6 ± 0.7 years. Both control and methadone maintenance subjects were free of any other drug use or abuse, as determined by routine urine and blood analysis. All subjects were male, aged 20–40 years; none had recent infections, active inflammatory disease, or positive HIV or hepatitis B surface antigen (HbsAg) tests. None of the subjects included in the study took drugs affecting the immune system or had a history of neuropsychiatric disorders. Subjects participating in this study gave their written informed consent. The study protocol was reviewed and approved by the Committee on Human Rights in Human Experimentation of Mahidol University.

2.2. Lymphocyte isolation

Peripheral blood lymphocytes were obtained from 18 healthy volunteers (33.85 ± 5.27 years old) and 18 methadone maintenance subjects (30.16 ± 3.38 years old). Fresh whole blood (20 ml) was drawn from the cubital vein and collected in plastic tubes containing ethylene-diaminetetraacetic acid (EDTA) as an anticoagulant. The blood was diluted 1:1 in 0.9% NaCl or PBS (phosphate buffer saline) containing 2% fetal bovine serum, layered onto ficoll-paque and then separated by centrifugation at low speed (1500 rpm) at 25 °C for 30 min. The enriched lymphocyte layer (interphase layer) was collected into a conical tube on ice. The lymphocytes were washed twice with PBS or 0.9% NaCl, and the purity was checked by Wright's staining; platelet contamination was less than 4%. The cell pellets were used for membrane preparation and receptor binding, culture, or RNA extraction.

2.3. [^{3}H]naloxone binding on lymphocyte membranes

Enriched lymphocytes were resuspended using assay 50 mM Tris–HCl buffer (10 ml) and then homogenized twice for 10 s with a tissue homogenizer (Ultra turrax T25) at low speed (13,000 min^{-1}). This suspension was centrifuged twice at 48,000 × g for 20 min at 4 °C using a super-speed centrifuge (Avanti J-25-1, Beckman Coulter Inc., Palo Alto, CA). The supernatant was discarded, and the membrane pellet was kept at −80 °C until the receptor-binding assay was done.

The membrane pellet was resuspended in 20 ml of 50 mM Tris–HCl pH 7.4 that contained 1.2 mM $MgSO_4$, 2.5 mM KCl, 10 mM glucose, 1 mM EDTA, and 1 mg/ml of BSA. [^{3}H]naloxone or unlabelled ligand naltrexone was diluted in this buffer. The reaction mixtures contained a final volume of 500 μl Tris–HCl buffer and 0.5 mg/ml of lymphocyte membrane protein and [^{3}H]naloxone (0.5–10 nM). The reaction was incubated in the presence or absence of 10 μM naltrexone at 30 °C for 75 min. Each assay was performed in duplicate. The reaction was terminated by filtering through GF/B Whatman filters under vacuum. The filters were washed twice with ice-cold buffer (50 mM Tris–HCl, pH 7.4). Receptor-bound radioactivity trapped on the filters was counted using a liquid scintillation spectrometer. Specific [^{3}H]naloxone binding was 40–50% of total binding. The data were analyzed using non-linear least-square regression analysis (Graphpad Prism 3.0 software). The maximum number (B_{max}) and the dissociation equilibrium constant (K_d) values of [^{3}H]naloxone binding to the lymphocyte membrane were obtained.

2.4. Lymphocyte cultures

Duplicate cultures containing 1 × 10^6 cells in 2.5 ml of RPMI media/dish (Gibco-BRL, Gaithersburg, MD), 10% heat-inactivated fetal calf serum, 100 IU/ml penicillin, 100 mg/ml streptomycin, 1% L-glutamine, and 1 mM HEPES were seeded onto a 35-mm-diameter culture dish, and the cells were incubated at 37 °C for 48 h in a 5% CO_2 incubator. During the *in vitro* study dealing with methadone's effect on gene expression of mu- and delta-opioid receptors in lymphocytes, methadone (0.01, 1.00, and 100 μM) was added to the culture media. To study whether the effect could be blocked by an opioid antagonist, 100 μM naloxone (Sigma Chemical, St. Louis, MO) was added 45 min before the addition of methadone. To determine whether the methadone effect was mediated through the G_i-protein, the cell culture was pretreated with pertussis toxin (PTX, 50 ng/ml) for 4 h, and then methadone was added. After incubation for 48 h, the cells were harvested, collected, and used for RNA isolation.

2.5. Semi-quantitative reverse-transcriptase polymerase chain reaction (RT-PCR)

Total RNA was extracted from lymphocytes (1.5 × 10^6 cells) using TRIzol™ Reagent (Gibco-BRL, Gaithersburg, MD) based on the protocol recommended by the manufacturer. PCR primer sets for opioid receptors were designed against the nucleotide sequences of the human mu-opioid and delta-opioid receptor cDNAs available in Genbank (HSL29301, Mestek et al., 1995 and HSU07882, Knapp et al., 1994, respectively). The actin primer set was also designed and used as the internal control for the relative semi-quantitative study. The primer sequences were:

Primer names	Sequence	PCR product name/size (bp)
MOR1	5′ GGT ACT GGG AAA ACC TGC TGA AGA TCT GTG 3′	Mu/441
MOR2	5′ GGT CTC TAG TGT TCT GAC GAA TTC GAG TGG 3′	
DOR1	5′ CGG CCA CCA ACA TCT ACA TCT 3′	Delta1/703
DOR2	5′ ACG GGG TTG AGG CTG CTA TTG 3′	
DOR3	5′ ATC TTC AGG CTC ACC ATG AT 3′	Delta2/356
DOR4	5′ CGG TCC TTC TCC TTG GAG CC 3′	
Actin 1	5′ CCC AGA GCA AGA GAG GCA TC 3′	Actin/830
Actin 2	5′ CTC AGG AGG AGC AAT GAT CT 3′	

Total RNA extracted from lymphocytes was reverse transcribed and amplified by PCR in a single tube using the protocol described in our previous study (Chetsawang et al., 1999). For each RNA sample, three PCR reactions were initially performed using three pairs of primers, MOR1/MOR2, DOR1/DOR2 ("outer" primers), and Actin1/Actin2, to generate fragments designated as Mu, Delta1, and Actin, respectively. The total volume of the 25-μl reaction mixture was composed of 0.2 mM of dNTPs (dATP, dCTP, dGTP, and dTTP), 2 mM of $MgCl_2$, 20 pmol of each oligonucleotide primer, 0.75 U of Taq DNA Polymerase (Promega, Madison, WI), and 4 U of AMV Reverse Transcriptase (Promega), in buffer containing 50 mM KCl, 10 mM Tris–HCl, pH 9.0, and 0.1% Triton X-100. The reaction tube was initially incubated at 42 °C for 30 min to reverse transcribe RNA into cDNA; this incubation was followed by 22, 25, and 30 amplification cycles to generate fragments of Actin, Mu, and Delta1, respectively. Since there was very low band staining on the first amplification for delta receptor (Delta1), the Delta1 fragment was used as the template for generating the nested-PCR product named Delta2 using DOR3 and DOR4 primers ("inner" primers). The total reaction volume of 25 μl was composed of 10 μl of Delta1 fragment, 0.2 mM dNTPs, 2 mM $MgCl_2$, 20 pmol of oligonucleotide primers, 0.75 U of Taq DNA polymerase in buffer containing 50 mM KCl, 10 mM Tris–HCl, pH 9.0, and 0.1% TritonX-100. The PCR profiles were initially incubated at 95 °C for 5 min and then for 24 cycles.

After amplification, the PCR products were analyzed on a 1.5% agarose gel. DNA bands were visualized by 0.1% ethidium bromide staining under UV light. The density of the DNA bands was analyzed using the NIH Image 1.5f computer program.

2.6. Determination of protein

The concentration of protein in the lymphocyte membranes was determined by the Bradford method (Bradford, 1976) using bovine serum albumin as a standard.

2.7. Statistical analyses

Saturation curves were analyzed by the non-linear least-square regression analysis computer program, Graphpad Prism 3.0 software (Graph Software, San Diego, CA). The data are expressed as mean ± S.E.M. derived from 18-experiments. Statistical evaluation of the result was performed by using the Student's t-test to compare between controls and the methadone maintenance group.

All values obtained from culture studies were based on a minimum of six experiments. Data are presented as mean ± S.E.M. The statistical evaluation of the

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