Regulation of the Transcription of the Catechol-O-Methyltransferase Gene by Morphine and Epinephrine

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Abstract

Changes in relative mRNA expression of catechol-O-methyltransferase (COMT) in cells treated with morphine and epinephrine were measured to determine if these hormones could influence their metabolic enzymes.

We used microarray and real time polymerase chain reactions to measure relative changes in COMT expression. Neuroblastoma cells capable of morphine synthesis were used as well as astrocytoma, adenocarcinoma, and a macrophage-like cell line. Cell lines investigated all had elements of the endogenous morphine signaling system. Epinephrine was added to a catecholamine producing cell line.

Morphine down regulated COMT when compared with control values. Cells treated with epinephrine up regulated their COMT expression.

It appears that morphine has the ability to modulate its own synthesis via autocrine and paracrine signaling. The metabolic pathways for catecholamines and morphine also can be influenced by the terminal catecholamine, epinephrine.

INTRODUCTION

Variations of the expression of genes in the morphine biosynthetic pathway may have profound effects on human health. Morphine is an endogenous chemical messenger whose presence or absence has not been previously considered in this light. The variations of these genes may have an influence over the way people respond to cognitive and physical stress, especially because dopamine (DA) is a precursor. Mood disorders, such as, aggression, depression, schizophrenia, and attention deficit/hyperactivity disorder could be linked to mutations in the genes of the morphine biosynthetic pathway via their role in DA metabolism or via a direct role in the biosynthesis of morphine post-DA (Cohen & Pickar 1981; De Luca *et al* 2006; Halleland *et al* 2009; Kopeckova *et al* 2008; Lachman 2008; Schmauss & Emrich 1985; Yamano E *et al* 2008).

Polymorphisms in some of the genes in the morphine and catecholamine metabolic pathways such as, tyrosine hydroxylase, DOPA decarboxylase, dopamine beta hydroxylase, and monoamine oxidase have not been as well studied as catechol-O-methyltransferase (COMT) in terms of their effects on human health (Haavik *et al* 2008). COMT has been implicated in morphine biosynthesis in both invertebrates and vertebrates (Kream & Stefano 2006), making it highly significant, especially since it exhibits polymorphisms. The most studied COMT polymorphism is termed val/met 158. This polymorphism has a methionine substituted for a valine at amino acid 158 (Syvanen *et al* 1997). Ongoing studies are attempting to establish a link between this polymorphism and behavior (Lachman 2008). The effect of this polymorphism is a lowering of the activity of COMT and thus a slower metabolism of DA (Kunugi *et al* 1997; Syvanen *et al* 1997).

In this report, experiments are presented to demonstrate the presence of and regulation of the COMT gene in multiple cell types. Cells from the immune, endocrine, and nervous systems were evaluated after exposure to morphine, epinephrine, a morphine receptor antagonist (naloxone), and a β-adrenergic receptor antagonist (propranolol).

MATERIALS AND METHODS

Morphine and opiate receptor antagonist exposures

To explore the changes in gene expression caused by morphine, tissues or cells were exposed to morphine for different durations. Human neuroblastoma cells obtained from the American Type Culture Collection (ATCC) (Manassas, Va) designated, SH-SY5Y (ATCC # CRL-2266), were cultured in a 1:1 mixture of Eagle's minimum essential medium and F12 medium supplemented with 10% fetal bovine serum. SH-SY5Y cells are capable of producing morphine (Poeaknapo *et al* 2004). Cells from a patient with histiocytic lymphoma, U-937 or ATCC # CRL1593.2, were also obtained from ATCC. U-937 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum. The media and serum were purchased from Invitrogen (Carlsbad, Ca). All cells were incubated at 37 °C in a 5% CO_2 atmosphere. SH-SY5Y cells were grown to 80% confluency in a 75cm^2 flask and treated with morphine at 10^{-6} M for 4, 18 or 48h. Total RNA isolated from treated astrocytoma cells (U373) was provided as a gift from Dr. Yannick Goumon. U373 cells were treated with 10^{-6}M for 4h and 48h. U937 cells were cultured in the vertical position to a density of 10^6 cells/ml and treated in 75 cm^2 flasks containing 10 ml of media. The final morphine concentration for the U937 cultures was 10^{-7} M and the incubation time was 4h. U937 cells were also pre-treated for 10 minutes with 10^{-6}M naloxone prior to the morphine addition. All experiments were performed in triplicate.

Morphine and epinephrine exposures

The effects of morphine or epinephrine treatments on mRNA expression of morphine biosynthetic genes were investigated using a colonicadenocarcinoma cell line (ATCC # CCL-220). The cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and incubated at 37 °C in a 5% CO_2 atmosphere. Cells were grown to 90% confluency in 75cm^2 tissue culture flasks. Incubations with 10^{-6} M and 10^{-7} M morphine lasted 4h and 24h. Cells were also exposed to 10^{-9} M and 10^{-8} M epinephrine for the same time periods. The β-adrenergic receptor antagonist, propranolol, was also used in the 24h experiments. Propranolol was obtained from Sigma (St. Louis, MO) and was added 30 min prior to adding epinephrine. The final concentration of propranolol was 20μM. After incubation, total RNA was isolated and assayed as described below. All treatments were done in triplicate.

Gene Expression Assays

Total RNA was isolated and purified using the RNeasy mini kit (Qiagen, Valencia, CA). Pelleted cells were resuspended in 600 μL of RLT lysis buffer (Qiagen) and homogenized by passing the lysate 20 times through a 1 mL pipette tip. The samples were then processed according to the manufacturer's detailed instructions (Qiagen, Valencia, Ca). In the final step, the RNA was eluted with 50 μl of RNase-free water by centrifugation for 1 min at 13,000 g. The RNA was analyzed on a model 2100 bioanalyzer (Agilent, Santa Clara, CA) using a total RNA nanochip according to the manufacturer's protocol.

Microarrays

Applied Biosystems Human Genome Survey Arrays were used to construct and differentially analyze by strict statistical criteria transcriptional gene expression profiles of experimentally manipulated cells or tissues. Digoxigenin-UTP labeled cRNA was generated and linearly amplified from 1 μg of total RNA using Applied Biosystems Chemiluminescent RT-IVT Labeling Kit v 2.0 and manufacturer's protocol. Briefly, each microarray was first pre-hybridized at 55 °C for 1 hr in hybridization buffer with blocking reagent. 15 μg of labeled cRNA targets was fragmented into 100–400 bases by incubating with fragmentation buffer at 60 °C for 30 min, mixed with internal control target (ICT, 24-mer oligo labeled with LIZ fluorescent dye) and hybridized to each pre- hybridized microarray in a 1.5-ml volume at 55 °C for 19 hr. After hybridization, the arrays were washed with hybridization wash buffer and chemiluminescence rinse buffer. Enhanced chemiluminescent signals were generated by first incubating arrays with anti-digoxigenin- alkaline phosphatase, enhanced with chemiluminescence enhancing solution and finally adding chemiluminescence substrate. AB1700 Expression System software was used to extract assay signal, and assay signal to noise ratio values from the microarray images. The specific genes were considered detectable if the signal to noise ratio was greater than 3. To minimize number of falsely significant genes, the identified set of genes was filtered by assay signal-dependent fold change thresholds. To calculate the thresholds, same treatment assay signal ratios were ordered by assay signal intensity and binned in 10 equal sets. The interquantile ranges of the each bin were used as the fold change thresholds. Genes with fold change within the corresponding interquantile range were removed from the selected list of differentially expressed genes. Genes with p-value<0.05 were identified as candidates for significantly differentially expressed genes. Microarrays were also performed using a system provided by Agilent. Arrays were constructed with the same oligo sequences as the Applied Biosystems' arrays and consisted of 4 arrays per chip (Agilent 4X44K chips). Total RNA was reverse transcribed (400 ng) using T7 primers and labeled and transcribed using Cyanine-3 dye. Each array was hybridized with at least 1.65 μg of labeled cRNA at 65 °C for 18h. Arrays were scanned using an Agilent array scanner. A 10% or greater change in gene expression could be determined using both microarray platforms.

Taqman Low Density Arrays-Real Time Polymerase Chain Reactions

First-strand cDNA synthesis was performed using random primers (Invitrogen, Carlsbad, CA). 2 μg of total RNA isolated from cells was denatured at 95 °C and reverse transcribed at 40 °C for 1 hr using Superscript III Rnase H-RT (Invitrogen, Carlsbad, CA). A Taqman low density array (Applied Biosystems) consisting of 11 genes of interest and 1 reference gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was used to evaluate the effects of morphine and epinephrine on COMT gene expression in colonicadenocarcinoma and morphine's effect on U937 cells. The array contained selected genes of the proposed morphine biosynthetic pathway. Prior to designing the low density array, an experiment was performed to determine the optimum reference gene. Human cells were treated for 4h with morphine, naloxone, and naloxone plus morphine and subjected to a reverse transcription-real time PCR with an Applied Biosystems Taqman low density array (endogenous control array, part # 4366071). GAPDH was selected as the most stable reference gene. For the arrays, 2 μg of total RNA was reverse transcribed and 1 μl of cDNA was diluted with 49 μl of H_2O. This diluted cDNA was mixed with 50 μl of 2X universal master mix (Applied Biosystems). The resulting mixtures were added to the appropriate sample ports on the array (8 samples per array). Arrays were run on the 7900HT fast real time PCR system (Applied Biosystems) using the default thermal cycler settings. Gene expression fold changes were determined by the software using the ΔΔCt method. Each PCR was performed in quadruplicate. A fold change of 1 indicated no change in expression. A fold change of 2 was considered to be statistically significant at the 99.75% confidence interval.

RESULTS

Microarrays

The effect of morphine exposure on COMT gene expression in neuroblastoma cells was evaluated at 4h, 18h, and 48h after exposure to 1μM morphine. The 4h treatment lowered the COMT expression by 7%, 18h treatment lowered it by 44%, and the 48h exposure lowered it by 13% (Fig. 1).

The effect of morphine exposure on COMT gene expression in astrocytoma cells was evaluated at 4h and 48h after exposure to 1 μM morphine. COMT was down by 28% at 4h and by 33% at 48h (Fig. 2).

Taqman Assays

The expression of COMT was evaluated by Taqman low density array in U937 cells (real time PCR). The PCR data revealed a 14% down regulation of COMT upon exposure to 10^{-7} M morphine for 4h (Fig. 3). Naloxone pretreatment was able to block this effect (data not shown).

Taqman low density arrays were used to measure changes in morphine biosynthetic gene expression in a colonicadenocarcinoma cell line after being incubated in the presence of morphine or epinephrine. Morphine treated cells had nearly a 6 fold decrease in COMT after 24h at both concentrations (10^{-6} and 10^{-7} M) evaluated (Fig. 4). The expression of this gene was also measured after 4h morphine exposure. After 4h, COMT did not change (Fig. 4).

Colonicadenocarcinoma cells treated with 10^{-9} M epinephrine for 24h showed a 1.6 fold increase in COMT expression (Fig. 5). Cells treated with 10^{-8} M epinephrine had a 1.3 fold increase in COMT expression after 24h (Fig. 5). The expression of this gene was also measured after 4h epinephrine exposure. COMT expression was unchanged with the higher dose of epinephrine, but the 10^{-9} M treatment up regulated it 1.7 fold (Fig. 5). When propranolol was used prior to epinephrine addition, the gene expression was unchanged (data not shown).

DISCUSSION

The present report provides evidence for the presence of COMT in numerous human cell lines. Exposing cells to morphine resulted in the down regulation of the COMT gene at 4h, 18h and at 48h. These findings were in agreement with the finding that morphine treated human leukocytes had a lower COMT expression (Mantione *et al* 2008). Epinephrine addition to the colonicadenocarcinoma cells increased COMT expression after 24h exposure and this effect was abolished by the β-adrenergic antagonist, propranolol. The increased COMT expression by epinephrine may be a means for catecholamines to interact with the morphine biosynthetic pathway and serve to signal morphine production over time. In addition, because of the ability of COMT to transform DA and epinephrine into relatively inactive metabolites, epinephrine could also be removed by the potentially increasing COMT levels. Morphine consistently down regulated COMT mRNA expression in this study. Nitric oxide has also been shown to down regulate COMT (Mantione *et al* 2008). Epinephrine can also stimulate NO production (Figueroa *et al* 2009), however because of the increased COMT in response to epinephrine this was most likely not occurring to any great extent. Thus, morphine appears to be inhibiting its own synthesis and may be affecting the catabolic enzyme for catecholamines. Epinephrine may be promoting its own catabolism or could be stimulating morphine production. COMT will not only transform DA so that the DA is unavailable to be utilized for morphine biosynthesis, but COMT is also necessary for methylation of morphine precursor molecules.

It is important to note the linking of morphine biosynthesis to that of DA (Kream & Stefano 2006; Stefano *et al* 2007; Stefano & Kream 2007). This coupling to dopaminergic processes has important biomedical significance. For example, initial speculation as to the existence and potential physiological role of endogenous morphine were made over 30 years ago by prominent researchers in the field of alcohol abuse, not opiate abuse, who advanced the hypothesis that the reinforcing or additive effects of ethanol were functionally linked to the cellular effects of DA derived isoquinoline alkaloids, notably the tetrahydroisoquinoline salsolinol (Davis *et al* 1970; Davis & Walsh 1970; Yamanaka *et al* 1970) and the benzylisoquinoline morphine precursor tetrahydropapaveroline (THP) (Halushka *et al* 1970; Walsh *et al* 1970; Weiner 1978). Recognition of tetrahydroisoquinolines, THP, and endogenous morphine as active principles of alcohol abuse was inherently linked to their normal presence in dopaminergic neurons, enhanced cellular expression following chronic ethanol intake (Collins *et al* 1979; Turner *et al* 1974; Weiner 1978; Weiner 1981; Zhu *et al* 2006a; Zhu *et al* 2006b), and concentration-dependent disregulation of DA metabolism and/or dopaminergic signaling in mesolimbic/mesocortical areas such as the nucleus accumbens and the ventral tegmental area traditionally associated with reward and reinforcement of ethanol intake (Clow *et al* 1983; Duncan & Fernando 1991; Myers 1990; Myers & Robinson 1999; Sallstrom *et al* 1999). The causal relationship and functional association of central nervous system expression of tetrahydroisoquinoline and benzylisoquinoline alkaloids to alcohol abuse remains controversial despite anatomical, physiological, pharmacological, and behavioral evidence linking dopaminergic and opioidergic systems in limbic areas associated with reinforcement of ethanol intake behaviors (Haber *et al* 1997; McCoy *et al* 2003; Naoi *et al* 2004; Shearman & Herz 1983).

This link is equally important when considering animal behavior. It can be surmised that the DA component modulates excitatory states, including rage, whereas the morphinergic component offers calming action associated with relaxation and reward. This association may also explain the calming effect following excitatory emotional states. Moreover, in this scenario of DA synthesis coming before that of morphine one would predict excitation would precede the calm, which may be associated with morphine signaling. Furthermore, this coupling may also explain the fact that within various relaxation techniques an excitatory stress component emerges physiologically before relaxation sets in (Stefano *et al* 2001). The link between catecholamine and morphine metabolism promises to be the subject of future investigations given its significance in biomedicine.

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