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Cisplatin causes over-expression of tachykinin NK_1 receptors and increases ERK1/2- and PKA phosphorylation during peak immediate- and delayed-phase emesis in the least shrew (*Cryptotis parva*) brainstem

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ABSTRACT

Scant information is available regarding the effects of cisplatin on the expression profile of tachykinin NK_1 receptors and downstream signaling during cisplatin-induced emesis. Cisplatin causes peak early- and delayed-phase emesis in the least shrew at 1–2 and 33 h post-injection . To investigate the expression profile of NK_1 receptor during both emetic phases, we cloned the cDNA corresponding to a ~700 base pairs of mRNA flanked by two stretches of nucleotides conserved among different species and demonstrated that the shrew NK_1 receptor nucleotide sequence shares ~90% sequence identity with the human NK_1 receptor. Of the 12 time-points tested, significant increases in expression levels of NK_1 receptor mRNA in the shrew brainstem occurred at 2 and 28 h post-cisplatin injection, whereas intestinal NK_1 receptor mRNA was increased at 28 h. Shrew brainstem and intestinal substance P mRNA levels also tended to increase during the two phases. Furthermore, expression levels of NK_1 receptor protein were significantly increased in the brainstem at 2, 8, and 33 h post-cisplatin . No change in brainstem 5-HT $_3$ receptor protein expression was observed. The temporal enhancements in NK_1 receptor protein expression were mirrored by significant increases in the phosphorylation status of the brainstem ERK1/2 at 2, 8, and 33 h post-cisplatin . Phosphorylation of PKA significantly increased at 33rd and 40th hour. Our results indicate associations between cisplatin's peak immediate- and delayed-phase vomiting frequency with increased: (1) expression levels of NK_1 receptor mRNA and its protein level, and (2) downstream NK_1 receptor-mediated phosphorylation of ERK1/2 and PKA signaling.

1. Introduction

Chemotherapeutics such as cisplatin produce immediate and delayed vomiting in humans and vomit-competent animals (Darmani et al., 2009; Hesketh et al., 2003; Rudd and Andrews, 2005). The current antiemetic regimens against chemotherapy-induced vomiting (CIV) are based upon the dogma that during the acute phase cisplatin releases serotonin (5-hydroxytryptamine= 5-HT) from the enterochromaffin cells in the gastrointestinal tract (GIT), which subsequently stimulates 5-HT $_3$ receptors on vagal afferents to initiate the vomiting reflex (Rudd and Andrews, 2005). The delayed phase emesis was thought to be due to activation of tachykinin NK_1 receptors subsequent to the release of substance P in the brainstem (Andrews and Rudd, 2004). In fact, 5-HT $_3$ receptor antagonists show significant anti-emetic efficacy during the acute CIV, whereas NK_1 receptor antagonists improve the antiemetic efficacy of conventional antiemetic drugs during the delayed phase CIV (Hesketh et al., 2003). However, complete protection against CIV has not yet been achieved. Our inability to develop more effective antiemetic regimens is due to incomplete appreciation of the relative contribution of multiple emetic transmitters and their temporal interplay in the regulation of both phases of CIV both in the brainstem and the GIT (Darmani et al., 2011; Darmani and Ray, 2009). To bridge some of the existing gaps, we have established the least shrew (*Cryptotis parva*) model of cisplatin-induced emesis. Indeed, a 10 mg/kg dose of cisplatin can induce both early (maximum frequency at 2 h)- and delayed (maximum frequency at 33 h)-phases of CIV in this species (Darmani et al., 2009).

Serotonin and substance P are major emetic neurotransmitters involved in emesis (Darmani and Ray, 2009; Margolis and Gershon, 2009). Serotonin exerts its effects via multiple serotonergic receptors of which 5-HT $_3$ receptors gate an ion channel permeable to monovalent and divalent cations (Adayev et al., 2005). The tachykinin neuropeptides include substance P, tachykinin A and tachykinin B which exert their biological effects via NK_1-, NK_2- and NK_3- receptors (Saria, 1999). Although substance P can bind to all tachykinin receptors, it has the highest affinity for the NK_1 receptor. Activation of NK_1 receptor can lead to diverse post-receptor signals such as the

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activation of phospholipase C (PLC) and adenylate cyclase cascade systems. Further downstream NK_1 receptor signaling includes activation of extracellular signal-regulated protein kinase1/2 (ERK1/2) and protein kinase A (PKA) cascades (Ramnath et al., 2007). Both 5-HT $_3$- and NK_1-receptors are key players in CIV (Darmani and Ray, 2009; George et al., 2010). The present study was undertaken to examine cisplatin-induced changes during the early and delayed phases of CIV in the expression levels of: (1) substance P and NK_1 receptor mRNAs, (2) NK_1- and 5-HT $_3$-receptor proteins, and (3) some proteins (e.g. ERK1/2 and PKA) that are associated with downstream signaling pathways of the NK_1 receptor. Since the genome sequence of the least shrew is not fully known, we cloned a portion of the shrew NK_1 receptor gene and used this sequence to design primers for profiling its expression pattern during the immediate and delayed phases of emesis.

2. Material and methods

2.1. Animals and treatment protocols

Male and female shrews were bred and housed in our animal facilities. Shrews weighing 4–6 g (45–70 days old) were used throughout the study. The animals were kept on a 14/10 h light/dark cycle at a room temperature of 22 ± 1 °C in a humidity-controlled environment with ad lib supply of food and water. All animals received care according to the Guide for the Care and Use of Laboratory Animals (Department of Health and Human Services Publication, revised, 1985). All of the procedures used in this study were approved by the Institutional Animal Care and Use Committee of the University. Cisplatin [cis-platinum (II) diamine dichloride ($Pt(NH_3)_2)Cl_2$] (Sigma, St. Louis, MO) was dissolved in saline by sonication and administered at a volume of 0.1 ml/10 g body weight.

2.2. Cloning of the shrew NK_1 receptor

Two regions of NK_1 receptor sequence were chosen based on their conservation in mouse, rat, tree shrew, chimpanzee and human gene sequences. Total RNA isolated from the least shrew was reverse transcribed by using gene-specific primer (TGTCATCTGGGTCCTGGCTCTCCTGCTGGCCTT) and the SuperScript™ III reverse transcription kit (Invitrogen) as per the protocol recommended by the manufacturer. An aliquot of the first strand cDNA was used for amplification by using a forward primer (GCCCACAAGAGAATGAGGACAGTGAC) and the reverse primer described above. The amplified product was separated on a 1.5% agarose gel and the 687 base pairs fragment was purified. The purified fragment was then ligated to TOPO TA cloning vector (Invitrogen) as recommended by the manufacturer. The ligated mixture was used to transform DH5α competent cells and the transformed cells were plated on agarose plates in the presence of ampicillin. Ten colonies were picked up from the plate, and grown in LB medium in the presence of ampicillin. The bacterial culture was processed for purifying the plasmid and the recombinant plasmid DNA was confirmed by the presence of the desired size DNA insert. The plasmid DNA from 5 independent clones was sequenced by the Sanger's method of dyedeoxy DNA chain termination. The sequences were then analyzed by comparison among themselves and with known sequences from other species.

2.3. Reverse transcription and polymerase chain reaction (RTPCR)

Adult least shrews treated with cisplatin (10 mg/kg, i.p., $n=4$ per group) were rapidly anesthetized with isoflurane and decapitated at the indicated time points post-treatment (see figures). Brainstem and gut samples were collected on ice and stored at –80 °C. Total RNA was isolated by using Trizol (Invitrogen-Life Technologies, Carlsbad, CA) and reverse transcribed with the Superscript III kit (Invitrogen) according to manufacturer's protocol. Briefly, 4 μg of total RNA was heated to 65 °C for 5 min, cooled on ice for 1 min, and mixed with 4 μl 5X first-strand buffer, 1 μl random hexamers and dNTP. The reaction was incubated at 25 °C for 5 min and then mixed with the reverse transcriptase buffer, 1 μl 0.1 M DTT, 1 μl RNaseOUT™ recombinant RNase inhibitor (40 units/μl), and 1 μl of SuperScript™ III modified MMLV reverse transcriptase (200 units/μl). First strand cDNA synthesis was carried out at 50 °C for 60 min and then the reaction was terminated by heating at 85 °C for 5 min. Taqman probes specific for Tac1 (substance P), NK_1 receptor (Biosource) and 18S rRNA (Applied Biosystems, Foster city, CA) were used to quantify their expression levels as described below. Briefly, 10 μl of 2X TaqMan buffer, forward and reverse PCR primers (18 μM each), TaqMan probe (final concentration 250 nM), 18S rRNA forward and reverse primers (18 μM each), VIC probe and 1 μl of cDNA were mixed in a final volume of 20 μl. The mixture was denatured at 95 °C for 10 min in a MiniOpticon and the transcripts were amplified for 40 cycles under the following conditions: 15 s at 95 °C, 15 s at 54 °C, and 40 s at 60 °C.

2.3.1. Primers and probe sequences

Tac1 Forward primer: 5′-AGCTGCCTGAGCCCTTTGAG-3′, Tac1 Reverse primer: 5′-GCATCACGTTTGCCCATCA-3′; Tac1 TaqMan probe: FAM-5′-CAGAGAATGGCCCGGAGACCCAAG-BHQ1-3′; NK_1 receptor forward primer: 5′-AGAATGAGGACAGTGAC-3′; NK_1 receptor reverse primer: 5′-AAGGCCAGCAGGAGAGCCAGG-3′; NK_1 receptor TaqMan probe: FAM 5′-ACGGTC TGTTCTACTGCAAGTTCCA-3′.

2.4. Western blotting

Brainstem tissues were homogenized with N-PER lysis buffer (Pierce) in the presence of protease inhibitors (Roche) and phosphatase inhibitor. Cellular debris was removed by centrifugation and the supernatant assayed for protein concentration by the BCA method (Pierce). Fifteen micrograms of protein was electrophoresed in a 12.5% NuPAGE tris-acetate mini-gel. The proteins were then transferred electrophoretically to a polyvinylidene difluoride membrane (Millipore Corporation, Billerica, MA). Membranes were incubated in 5% skim milk in phosphate-buffered saline containing 0.05% (v/v) Tween-20 (PBST) for 1 h at room temperature, then incubated separately at 4 °C overnight with one of the following antibodies: NK_1 receptor mouse monoclonal antibody (Invitrogen), 5-HT $_3$ rabbit polyclonal antibody (Thermo Scientific), extracellular signal-regulated protein kinase (ERK1/2) (3A7) mouse antibody (Cell Signaling), phospho- ERK1/2 (Thr202/Tyr204) rabbit antibody (Cell Signaling), PKA rabbit antibody (Cell Signaling), phospho-PKA rabbit antibody (Invitrogen), or anti-glyceraldehyde-3-phosphate dehydrogenase mouse monocolonal antibody (GAPDH) (Millipore). After incubation with antibodies, the membranes were washed three times with PBST, and then incubated with either peroxidase-labeled anti-mouse IgG antibody (Vector Laboratories) or peroxidase-labeled anti-rabbit IgG antibody (GE Health Care UK Limited). The membrane containing immunoreactive complexes was incubated with detection reagents (Amersham Biosciences Inc., Piscataway, NJ) according to the manufacturer's instructions, and then exposed to an X-ray film. The intensity of each protein band on the film was analyzed with the ImageJ software (NIH).

2.5. Statistical analyses

Data were expressed as Mean ± S.E.M. One-way analysis of variance (ANOVA) followed by Bonferroni test were used for