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Cyclophosphamide causes activation of protein kinase A (PKA) in the brainstem of vomiting least shrews (*Cryptotis parva*)

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ABSTRACT

Complete control of emesis caused by cyclophosphamide (CPA) is of immense interest to both patients and physicians. Serotonin 5-HT_3- and tachykinin NK_1-receptor antagonists are widely used antiemetics in clinic, but they fail to completely control CPA-induced emesis. New antiemetic targets for the full control of CPA-induced vomiting are lacking. We therefore examined the effects of CPA on emetic targets downstream of 5-HT_3- and NK_1- receptors in an attempt to better understand the molecular bases of CPA-induced emesis. Acute CPA (200 mg/kg, i.p.) administration in the least shrew caused a biphasic pattern of emesis over a 40 h observation period, with maximal peak vomit frequency during the 1st hour of treatment (acute phase), followed by a delayed-phase which peaks at 27th hour. The NK_1 receptor mRNA levels increased significantly at 8 h post-CPA treatment in the brainstem, and at 28 h in the whole intestine. Substance P mRNA levels tended to increase both in the brainstem and intestine at most time-points post-CPA injection, however due to large variability, they failed to attain significance. Likewise, protein expression profiles of both NK_1- and 5-HT_3 -receptors in the brainstem were unchanged at any time-point. However, phosphorylation levels of protein kinase A (PKA), but not of extracellular signal-regulated protein kinase 1/2 (ERK1/2), were increased at 2, 8, 22, 28, and 33 h time-points after the treatment with CPA. Moreover, brainstem but not frontal cortex cAMP tissue levels tended to be elevated at most time-points, but significant increases occurred only at 1 and 2 h post-CPA treatment. The phosphodiesterase inhibitor, rolipram, caused significant increases in shrew brainstem cAMP levels which were associated with its capacity to produce vomiting, while pretreatment with SQ22536, an inhibitor of adenylyl cyclase, prevented rolipram-induced emesis. The results demonstrate that accumulation of cAMP and subsequent activation of PKA in the brainstem may help to initiate and sustain emesis induced by CPA in the least shrew. Our findings suggest that suppression of the cAMP/PKA cascade may have antiemetic potential in the management of CPA-induced emesis.

1. Introduction

Highly emetogenic chemotherapeutics, such as cisplatin, cause immediate and delayed vomiting in both 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 hypothesis 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 (Darmani and Ray, 2009; Rudd and Andrews, 2005). The delayed-phase emesis is 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; Darmani and Ray, 2009). In fact, 5-HT_3 receptor antagonists show significant antiemetic efficacy during cisplatin-induced acute CIV; whereas NK_1 receptor antagonists improve the antiemetic efficacy of conventional antiemetic drugs during the delayed phase CIV (Hesketh et al., 2003; Warr, 2012). Cyclophosphamide (CPA) is also a potent and highly effective chemotherapeutic which has been marketed for approximately 50 years. CPA-containing chemotherapy regimens are frequently given as outpatient treatment for various malignancies and are known to produce mild to severe nausea and vomiting in patients (Beck, 1995). As a pro-drug, CPA is inactive in vitro and exerts biologic activity though its metabolites generated by hepatic microsomal enzymes (Ahmed and Hombal, 1984). One of the main metabolites of CPA is phosphoramide mustard. Phosphoramide mustard is reported to induce serotonin (5-HT) release in the gastrointestinal tract (GIT) (Minami et al., 1997b)

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which ultimately contributes to vomiting (Fetting et al., 1982; Research, 2004). The involvement of serotonin 5-HT_3 receptors in the GI and brainstem in CPA-induced emesis is suggested from studies on cats and ferrets (Fetting et al., 1982; Hawthorn et al., 1988). The ablation of the chemoreceptor trigger zone in the brainstem of cats reduced, but did not abolish, the emetic response to CPA (Fetting et al., 1982). On the other hand, a combination of bilateral abdominal vagotomy and greater splanchnic nerve section in ferrets markedly reduced, but did not abolish, vomiting in response to intraperitoneal CPA (Hawthorn et al., 1988). Moreover, 5-HT_3 receptor antagonists such as ondansetron are effective in the clinic and laboratory against CPA-induced emesis in both patients and animal models of emesis (Beck, 1995; Beck et al., 1993; Crucitt et al., 1996). As with cisplatin, antagonists of substance P (SP) NK_1- and dopamine D_2 receptors are also varyingly effective against CPA-induced emesis in animals (Gardner et al., 1995; Gylys et al., 1988). Furthermore, combination of a 5-HT_3 (palonosetron)- and an NK_1 (aprepitant)-receptor antagonist with dexamethasone (a synthetic glucocorticoid) provides better emesis protection in cancer patients receiving CPA (Grunberg et al., 2009).

Currently, serotonin and SP are recognized as the major emetic neurotransmitters involved in the induction of CIV (Darmani and Ray, 2009). Although evidence for the involvement of 5-HT_3- and NK_1-receptors and development of their selective antagonists were respectively envisaged over 2 to 3 decades ago, nausea and vomiting still remain a significant problem for cancer patients (Grunberg et al., 2010). Since release of multiple emetic transmitters are involved in CIV (Darmani and Ray, 2009), we recently investigated the role of common molecular targets shared downstream of diverse emetic receptors following cisplatin administration in the least shrew (Darmani et al., 2013). We found significant associations between peak immediate and delayed vomiting with increased phosphorylation of ERK1/2 (extracellular signal-regulated protein kinase 1/2) and PKA (protein kinase A). These enzymes are part of the two signaling cascades of phospholipase C (PLC) and PKA, which can be activated by stimulation of emetic receptors, including NK_1 receptors (Ramnath et al., 2007). Thus, in the current study we initially investigated the possible biphasic emetic repertoire of CPA in the least shrew. Subsequently we determined whether CPA administration induces changes in the least shrew brainstem levels of: (1) Substance P mRNA, (2) NK_1- receptor mRNA and corresponding receptor protein, (3) 5-HT_3 receptor protein, (4) phosphorylation (activation) of ERK1/2 and PKA, and (5) cAMP.

2. Materials and methods

2.1. Animals and treatment

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 in a humidity-controlled environment at a room temperature of 22 ± 1 °C 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.

2.2. Drugs

Cyclophosphamide monohydrate (CPA), rolipram and SQ22536 (Sigma, St. Louis, MO) were dissolved in saline. The drugs were administered at a volume of 0.1 ml/10 g body weight.

2.3. Behavioral procedures

To habituate the shrews to the test environment, animals were randomly selected and individually-housed in a $14 \times 17 \times 12$ cm clean opaque plastic cage and were offered 10 mealworms 1 h prior to experimentation. Based upon our published cisplatin studies in the least shrew and published CPA studies in other emesis models (e.g. Gardner et al., 1995), as well as our preliminary experiments, 2 different groups of least shrews received either a single intraperitoneal (i.p.) injection of CPA (200 mg/kg) or a single i.p.-injection of saline at 9:00 AM. Immediately following injection, each shrew was returned to its cage and their behaviors were videotaped by video cameras (Panasonic color CCD camera, WV-CP450) which were mounted 30 cm above every cage and emetic behaviors were scored for up to 40 h post-injection. During the dark phase, a red light bulb above the video cameras was used to illuminate the shrew cages. During the entire experiment, animals were freely allowed food (mealworms) and water. Food supply was maintained by making sure that meal worms were always available in the cage throughout the observation period. Water was available throughout the experiment via a small plastic container (1 cm in diameter and 0.5 cm in height) glued to the floor of each cage. The mean frequencies ($\pm$S.E.M.) of vomiting (wet, dry and total) were scored separately from videotape recordings for each individual shrew for the entire observation period by a trained observer who was blind to the experimental conditions.

2.4. Reverse transcription and polymerase chain reaction (RT-PCR)

Adult least shrews treated with CPA (200 mg/kg, i.p., $n=3$ per group) were rapidly anesthetized with isoflurane and decapitated at the indicated post-treatment time points (see Fig. 2). Brainstem and gut samples were collected on ice and stored at −80 °C until use. Total RNA was isolated by using Trizol (Invitrogen-Life Technologies, Carlsbad, CA) and reverse transcribed with the Superscript III kit (Invitrogen) according to the 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 5× 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 2× 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, 40 s at 60 °C. 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′.

2.5. Western blot

Shrews were decapitated at the indicated time points. Brainstem samples were collected on ice and were kept at −80 °C