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Characterization of Substance P processing in mouse spinal cord S9 fractions using high-resolution Quadrupole-Orbitrap mass spectrometry

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ABSTRACT

Tachykinins are a family of pronociceptive neuropeptides with a specific role in pain and inflammation. Several mechanisms regulate endogenous tachykinins and Substance P (SP) levels, including the differential expression of protachykinin mRNA and the controlled secretion of tachykinins from neurons. Proteolysis is suspected to regulate extracellular SP concentrations but few studies were conducted on the metabolism of proneuropeptides and neuropeptides. Here, we provide evidence that proteolysis controls SP levels in the spinal cord leading to the formation of active C-terminal fragments. Using high-resolution mass spectrometry, specific tachykinins fragments were characterized and quantified. The metabolic stability of β-Tachykinin$_{58–71}$ and SP were very short resulting in half-life of 5.7 and 3.5 min respectively. Several C-terminal fragments were identified, including $SP_{3–11}$, $SP_{5–11}$ and $SP_{8–11}$, which conserve affinity for the Neurokinin 1 receptor. Interestingly, the metabolic stability of C-terminal fragments was significantly superior. Two specific Prolyl endopeptidase inhibitors were used and showed a significant reduction in the rate of formation of $SP_{3–11}$ and $SP_{5–11}$ providing strong evidence that Prolyl endopeptidase is involved into N-terminal processing of SP in the spinal cord.

1. Introduction

The substance P (SP) belongs to the tachykinin family and it is one of the most extensively studied neuropeptides. SP is widely distributed within the central nervous system (CNS) and it is a recognized neurotransmitter and a modulator of pain (Gao and Peet, 1999; Ribeiro-da-Silva and Hokfelt, 2000; Pailleux et al., 2013). The tachykinin precursor 1 (Tac1) gene encodes the protachykinin-1 protein containing the sequence of four tachykinin peptides, including SP (Cao et al., 1998; Basbaum, 1999). The protachykinin-1 protein is believed to be cleaved by the action of specific proteases into active neuropeptides by post-translational proteolytic processing during axonal transport (Hook et al., 2008). More specifically, recent studies outlined the significant contribution of the prohormone convertases PC1/3 and PC2 in the C-terminal processing of protachykinin peptides (Miller et al., 2003) and suggest a major role in the maturation of the protachykinin-1 protein (Saidi et al., 2015). Specifically, PC1/3 and PC2 are important in the synthesis of β-tachykinin$_{58–71}$, a known precursor of SP (Pailleux et al., 2013). The β-tachykinin$_{58–71}$ (Tach$_{58–71}$) is then processed to SP by the action of specific carboxypeptidases E (CPE) (Fricker, 2005; Hook et al., 2008), following C-terminal amidation by peptidylglycine-α-amidating mono-oxygenase (PAM) leading to the biosynthesis of SP (Fig. 1). Several mechanisms regulate endogenous tachykinins and SP levels, including the differential expression of protachykinin mRNA and the controlled secretion of tachykinins from neurons. Proteolysis is suspected to regulate extracellular SP concentrations (Mitchell et al., 2013). It is well known that upon their release, SP can be attacked, cleaved and inactivated by numerous enzymes (Snijdelaar et al., 2000). Several enzymes were suspected to be involved in the conversion of SP to specific N- and C-terminal fragments. Moreover, the action of some of these enzymes will lead to SP fragments preserving noticeable biological activities (Fig. 1). In a previous study, we have shown the up-regulation of two major C-terminal SP fragments, $SP_{3–11}$ and $SP_{5–11}$ using a rat model of neuropathic pain (Pailleux et al., 2013).

Abbreviations: Tach$_{58–71}$, β-tachykinin $_{58–71}$; SP, Substance P; $SP_{3–11}$, Substance $P_{3–11}$; $SP_{5–11}$, Substance $P_{5–11}$; TAC1, Tachykinin precursor 1; NK1, Neurokinin 1 receptor; CNS, Central nervous system; PC1/3, Prohormone convertases 1/3; PC2, Prohormone convertases 2; CPE, Endopeptidases E; PAM, Peptidylglycine monooxygenase; PREP, Prolylendopeptidases; TFA, Trifluoroacetic acid; MS, Mass spectrometry; MS/MS, Tandem mass spectrometry; HPLC, High performance liquid chromatography; IDMS, Isotope dilution mass spectrometry; ESI, Electrospray ion source; TIC, Total ion chromatogram; XIC, Extracted ion chromatogram; HRAM, High-resolution, and accurate-mass; FWHM, Full width at half maximum.

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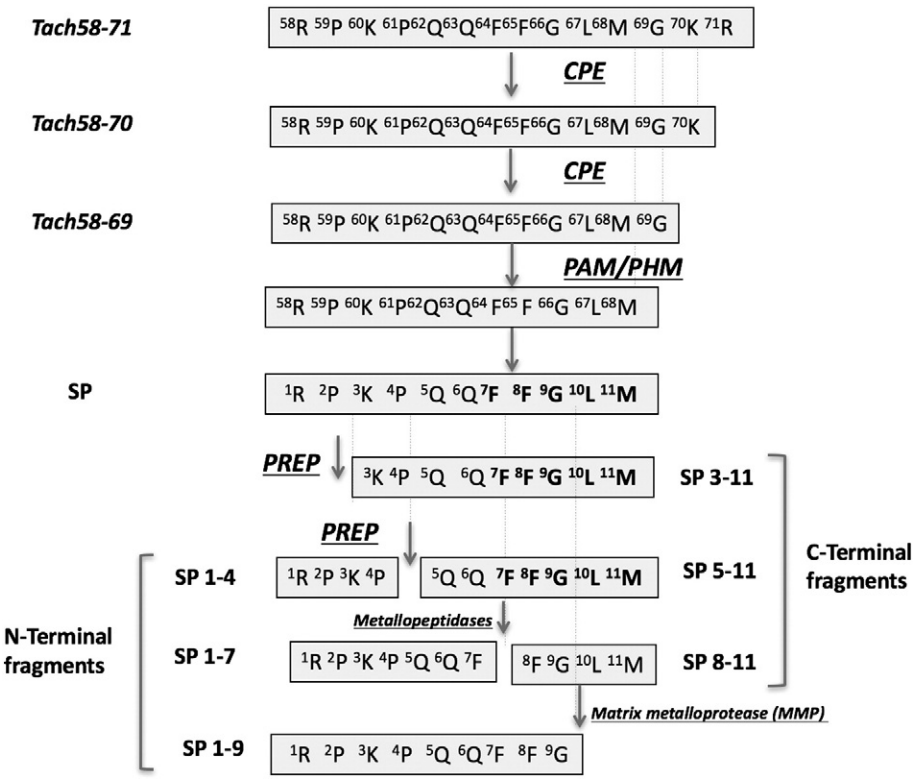

Fig. 1. N-terminal and C-terminal processing of $Tach_{58-71}$ and SP.

Both fragments can result from the N-terminal processing of $Tach_{58-71}$ or SP by the action of proline-specific endopeptidases prolylendopeptidases. As previously shown, the prolylendopeptidases (PREP) catalyzed the hydrolysis of Pro-X bond of peptides with primary sequence shorter than 30 amino acids (Polgár, 2002; Nolte et al., 2009). The expression of PREP mRNA in the spinal cord was described and it was suggested that tachykinin peptides can be hydrolyzed by PREP (Nolte et al., 2009). Enzyme catalyzing reaction yield and rate heavily depends on the thermodynamic stability of the enzyme-substrate complex (Berg et al., 2002). The reaction leading to the formation of SP_{3-11} and SP_{5-11} required the formation of two distinct enzyme-substrate complexes implying that one structure is probably thermodynamically more favorable compared to the other. It is known that C-terminal SP fragments play an important role in the overall SP pronociceptive effect favoring the release of the excitatory amino acids (Skilling et al., 1990). However, SP and C-terminal SP fragments have distinctive binding affinities with the NK1 receptor (Vasiliou et al., 2007; Mistrova et al., 2016) and the resulting effect will depend greatly on the relative abundance of metabolite fragments. Agonists of NK1 receptors affect the postsynaptic membrane and provoke a sustained slow depolarization that significantly contributes to the development of secondary hyperalgesia. The relative expression of $Tach_{58-71}$, SP, SP_{3-11} and SP_{5-11} will inherently affect pain transmission mechanisms, including pronociceptive and most likely antinociceptive actions. Consequently, not only SP should be considered, but the SP precursor $Tach_{58-71}$ and SP metabolites can have important functions during the development of secondary hyperalgesia.

Our main hypothesis is that proteolysis controls physiological SP levels in spinal cords leading to the formation of active C-terminal SP fragments. The objectives of this study were initially to develop and validate a HPLC–MS/MS assay to characterize and quantify specific tachykinins peptides. The secondary objective was to assess the metabolic stability of $Tach_{58-71}$, SP and peptide fragments. The tertiary objective was to determine the role of PREP in N-terminal processing of $Tach_{58-71}$ and SP in the spinal cord by using two specific Prolyl endopeptidase inhibitors.

2. Materials and methods

2.1. Chemicals and reagents

β-$Tachykinin_{58-71}$ ($Tach_{58-71}$), β-$Tachykinin_{58-70}$, Substance P (SP), Substance P_{3-11} (SP_{3-11}), Substance P_{5-11} (SP_{5-11}), Substance P_{8-11} (SP_{8-11}), Substance P_{1-9} (SP_{1-9}) and Substance P_{1-7} (SP_{1-7}) were purchased from Phoenix Pharmaceuticals (Belmont, CA, USA). Deuterium labeled analogue peptides were synthesized (CanPeptide, Inc., Pointe-Claire, QC, Canada). Formic acid, water (HPLC-MS Optima grade), acetonitrile (HPLC-MS Optima grade), hexane, trifluoroacetic acid (TFA), sodium phosphate dibasic and sodium phosphate monobasic were purchased from Fisher Scientific (NJ, USA).

2.2. Sample preparation

Spinal cord tissues (n = 6 per) from male wild type (C57BL/6J) were obtained from The Jackson Laboratory (Bar Harbor, Maine, USA) and kept frozen at −80 °C until analysis. All mice were 8 weeks old at time of tissues collection. The animals were euthanized with an overdose of isoflurane followed by a transection of the cervical spine. A flush of saline was performed within the spinal canal to collect the spinal cord lumbar enlargement. Tissue samples were snap-frozen in cold hexane (−60 °C) and stored immediately at −80 °C pending analyses. The study protocol was approved by the Institutional Animal Care and Use Committee of the Faculty of Veterinary Medicine of the University of Montreal and it was performed in accordance with the guidelines of the Canadian Council on Animal Care.

All six spinal cords were pooled and homogenized in a 0.1 M phosphate buffer, pH 7.4 at a ratio of 1:5 (w:v). Samples were sonicated 20 min and the homogenates were centrifuged at 9000*g* for 20 min. The total amount of protein in each supernatant was determined using the standard Coomassie protein assay (Bradford). This procedure was necessary in order to assure the same amount of protein was used for each experiments. Supernatant aliquots, designated as S9 fractions,