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BRIEF COMMUNICATION

Nociceptin In Vitro Biotransformation in Human Blood

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YU, J., B. T. CHAIT, L. TOLL AND M. J. KREEK. *Nociceptin in vitro biotransformation in human blood.* PEPTIDES **17**(5) 873–876, 1996.—In vitro biotransformation of a newly sequenced neuropeptide of 17 amino acid residues, named nociceptin and orphanin FQ by two separate research groups, was studied in human blood using matrix-assisted laser desorption/ionization mass spectrometry. Processing was carried out in freshly drawn blood incubated at 37°C for various time periods. It was found that cleavage at peptide linkage Phe[1]-Gly[2] was the predominant biotransformation pathway. Nociceptin(2–17) was the major biotransformation product. Further processing also occurred with the formation of a variety of minor biotransformation products. Cleavages at basic amino acid residues were observed, although these were not major biotransformation pathways found under these in vitro experimental conditions. Biotransformation of nociceptin followed a similar pattern to that of another neuropeptide, the endogenous opioid dynorphin A(1–17), but it appeared that nociceptin was more resistant to biotransformation in human blood in vitro than dynorphin A(1–17).

Biotransformation Nociceptin Orphanin FQ Blood Neuropeptide

VERY recently, a new neuropeptide was isolated and sequenced by two independent research groups (11,14). This peptide has 17 amino acid residues whose sequence was determined to be Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-Leu-Ala-Asn-Gln. This sequence closely resembles dynorphin (Dyn) A(1–17) (Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln). Preliminary studies (11,14) indicate that this new neuropeptide does not bind to the classical μ-, δ-, and κ-opioid receptors. This heptadecapeptide binds to a G-protein-coupled opioid receptor-like ORL_1 receptor (or the so-called orphan receptor, which does not bind any endogenous opioids), which has a sequence similar to opioid receptors (3,4,8,10,12,16,17). This new neuropeptide exhibited potent inhibition of forskolin-induced accumulation of adenylyl cyclase in the recombinant CHO(ORL_1+) but not in the nonrecombinant CHO(ORL_1−) cell line (11,14). This peptide induces hyperalgesia when administered ICV to mice as shown by the hot plate assay (11) and the tail flick assay (14). This new neuropeptide was named nociceptin by one research group (11) according to its apparent pro-nociceptive properties; it was also named orphanin FQ by the other research group (14), which refers to its orphan receptor binding properties and the termini of its amino acid sequence. In this communication, we report the first study of in vitro biotransformation of this peptide in freshly drawn human blood.

METHOD

Freshly drawn blood was obtained from two healthy male human subjects, 25 and 33 years of age. Blood was drawn into vacutainer tubes preconditioned with EDTA (Becton Dickinson, Rutherford, NJ).

In the two processing experiments, 2 and 3.5 ml of the blood, respectively, were transferred to vacutainer tubes without EDTA precondition. Nociceptin [Noc(1–17)], in the amount of 0.5 mg/ml, was immediately added to the blood. This Noc-containing blood was incubated in a water bath (Bench Scale Equipment Co., Inc., Dayton, OH) at 37°C. In the experiment with 2 ml blood, four 500-μl samples were collected at the following time points after the addition of Noc(1–17): 0, 15, 30, and 60 min. In the experiment with 3.5 ml blood, seven 500-μl blood samples were collected from the incubating tube at the following time points after the addition of Noc(1–17): 0, 15, 30, 60, 120, 180, and 240 min. Immediately after removal from the incubating tube, the blood was centrifuged in a Sorvall RC-5B refrigerated (0–5°C) superspeed centrifuge (Sorvall Instruments, DuPont Company, Newtown, CT), at 4000 rpm for 5 min, to separate the plasma. Then 200 μl of the plasma containing the biotransformed Noc was added to 1.8 ml of 1% trifluoroacetic acid (TFA) aqueous solution. The samples were stored at −40°C until analysis.

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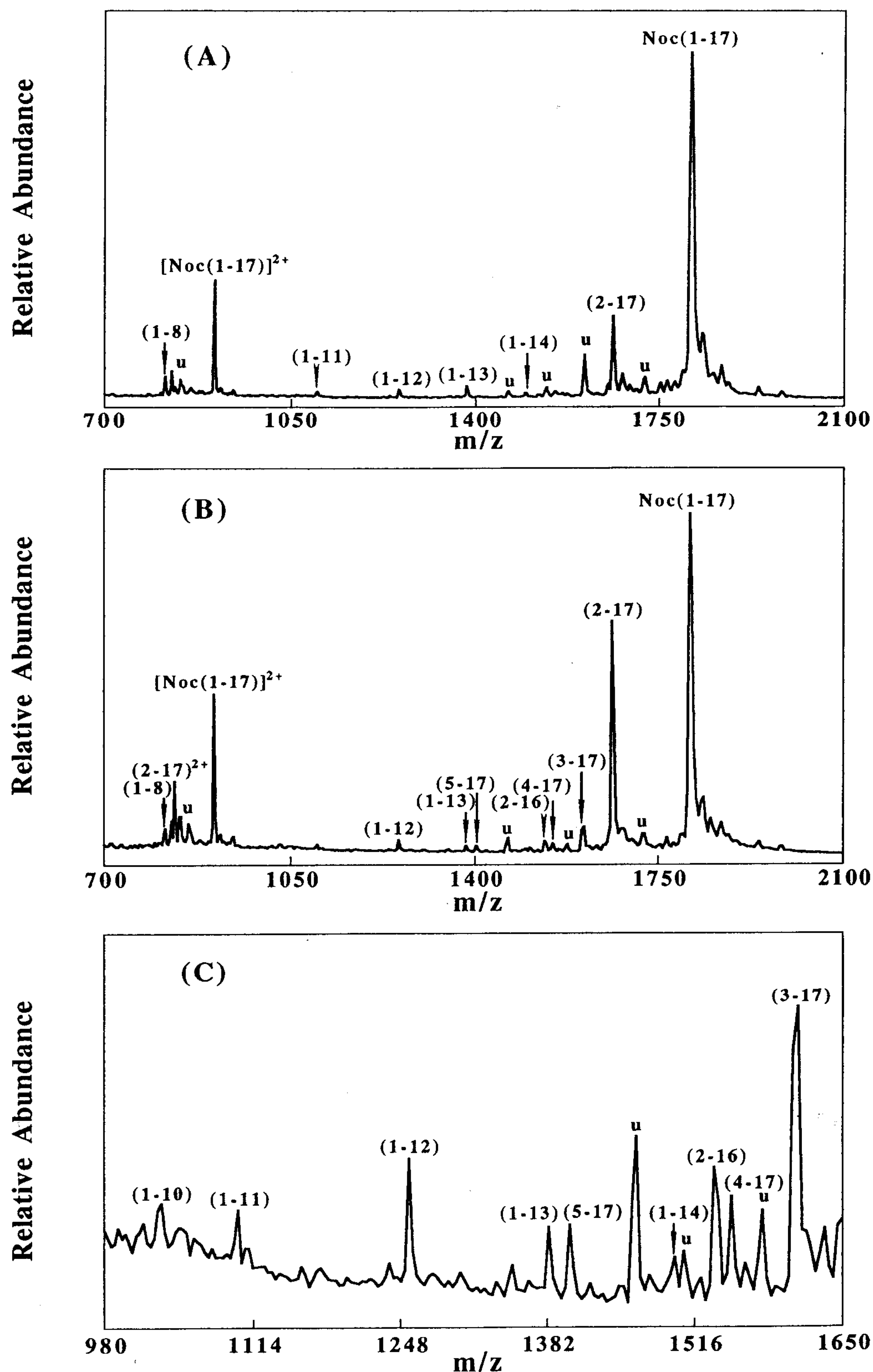

FIG. 1. Matrix-assisted laser desorption/ionization mass spectra of nociceptin(1–17) (0.5 mg/ml) in vitro biotransformation, incubation at 37°C in freshly drawn human blood of (A) male, 25 years, for 60 min; and (B) male, 33 years, for 240 min. (C) is from spectrum B in the *m/z* range of 980–1650. The numbers refer to nociceptin fragments; the notation of 2+, doubly charged ions; u, unidentified peaks.

TABLE 1

BIOTRANSFORMATION PRODUCTS OF NOCICEPTIN [Noc (1–17)] IN VITRO PROCESSING IN HUMAN (n = 2) BLOOD

(2–17)	(1–14)
	(1–13)
(3–17)	(1–12)
(4–17)	(1–11)
(5–17)	(1–10)
	(1–8)
(2–16)*	(1–16)*

The numbers refer to amino acid residues of the peptide fragments.

* (1–16) may be an impurity in the starting material. (2–16) may be a biotransformation product from (1–16).

Before analysis, the samples were thawed at room temperature, and were centrifuge filtered with Centricon-SR3 concentrators (Amicon Inc., Beverly, MA) with molecular weight cut-off of 3000 Da. After filtration, 100 μl of the filtrate was evaporated in a Speed Vac concentrator (Savant Instruments, Farmingdale, NY) for 20 min to reduce the total volume to approximately 40 μl.

Mass spectrometric analysis was performed with a matrix-assisted laser desorption/ionization linear time-of-flight mass spectrometer assembled at the Rockefeller University (1,2). For mass spectrometric measurement, 20 μl of the sample solution was mixed with 10 μl of acetonitrile (ACN). Plasma samples with ACN (2:1) were mixed with matrix solution. Matrix solution was made by dissolving excess amount (5 mg/ml) of α-cyano-4-hydroxycinnamic acid in ACN and 0.1% aqueous TFA (1:2, v/v) to make a saturated solution. The sample was mixed with matrix solution in a ratio of 1:2 (v/v). A 0.5-μl aliquot of sample/matrix solution was applied to the mass spectrometer sample probe tip, and allowed to evaporate to dryness in the air. The sample probe was inserted into the mass spectrometer for analysis. To obtain adequate statistics, the results from 200 laser shots were added for each mass spectrum.

RESULTS

Noc(1–17), upon in vitro incubation at 37°C in human blood, was biotransformed into a variety of Noc fragments. Table 1 summarizes the biotransformation products of Noc(1–17) detected in the blood from two human subjects. The major biotransformation product was Noc(2–17) (Fig. 1). This product was further processed into minor products: Noc(3–17), Noc(4–17), and Noc(5–17). Other minor biotransformation products, such as Noc(1–14), Noc(1–13), Noc(1–12), Noc(1–11), Noc(1–10), and Noc(1–8), were also detected (Fig. 1). Noc(2–16) may be a minor biotransformation product from Noc(1–17), or may be from the impurity, Noc(1–16), that was identified by our mass spectrometric method in the starting material.

DISCUSSION

Matrix-assisted laser desorption/ionization mass spectrometry permits the immediate and sensitive as well as specific detection of all major and most minor biotransformation products of the novel neuropeptide, nociceptin, in a biological system, and only very small amounts of neuropeptide are required. This technique has been used in our laboratory in several extensive studies of biotransformation of the natural opioid peptide, Dyn A(1–17), the shortened natural sequence peptide, Dyn A(1–13), and the synthetic analogue, Dyn A(1–10)-amide, by human and rhesus monkey blood in vitro and by rat brain tissues in vitro and in vivo using the combined technique of microdialysis in freely moving animals (5–7,18,19).

The biotransformation of Noc(1–17) was found to exhibit similar patterns to that of Dyn A(1–17) in vitro in human blood. Previous studies (5,6,18,19) of in vitro biotransformation have indicated that Dyn A(1–17) was biotransformed into a variety of Dyn A fragments. Two major biotransformation pathways were identified. One major pathway is the slow cleavage of the amino-terminal, tyrosine, to yield Dyn A(2–17). Dyn A(2–17) was further biotransformed into Dyn A(3–17) and Dyn A(4–17). However, this latter conversion occurred very slowly. Another major biotransformation pathway is the cleavage of the peptide linkage between Arg^6 and Arg^7 to produce Dyn A(1–6) and Dyn A(7–17).

In Noc(1–17) biotransformation during in vitro incubation in human blood, cleavage of the amino-terminal, phenylalanine, to yield Noc(2–17) was found to be a dominant biotransformation pathway. This was a slow process with prolonged presence of Noc(2–17) in the blood (Fig. 1). Noc(2–17) was further biotransformed into minor products: Noc(3–17), Noc(4–17), and Noc(5–17). The formation of minor biotransformation products may also arise from the actions of multiple enzymes present in the blood, which may result in nonsequential hydrolysis of the peptide.

Data accumulated in earlier studies (9) indicated that proteolytic cleavage of peptide and protein precursors (e.g., proproteins, prohormones, etc.) often occurs at paired basic amino acid residue sites. The most common pairs of basic residues are Lys-Arg, Arg-Lys, Arg-Arg, and Lys-Lys.

Cleavage of Noc(1–17) at paired basic residue sites was observed in the present studies. Cleavage of Noc(1–17) at peptide linkage Arg^8 and Lys^9 position with the formation of Noc(1–8) may be an important biotransformation pathway, although relatively very small amount of Noc(1–8) was detected, and no mass spectrometric signal arising from the complementary fragment, Noc(9–17), was found. The formation of Noc(1–11), Noc(1–12), Noc(1–13), and Noc(1–14) may arise from the presence of the paired basic residues Arg^{12}-Lys^{13}. The relatively small amount of proteolytic products formed from cleavages around basic amino acid residues may indicate that the enzymes responsible for these cleavages were not at optimal state under the experimental conditions.

Comparing the mass spectra of Noc(1–17) and Dyn A(1–17) at 240 min incubation, it appeared that Noc(1–17) was more resistant to biotransformation at the 1 to 2 and all the other positions than Dyn A(1–17) in human blood in vitro (18,19).

The biotransformation products, both the major and the minor, may be biologically active. The physiological effects from this neuropeptide may be the combination of the effects from the precursor peptide and metabolic fragments, as is the case with dynorphin A peptides (13,15).

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