

## Neutral Endopeptidase Activity in Serum and Cerebrospinal Fluid

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### ABSTRACT

We measured neutral endopeptidase (NEP) activity in serum from non-smoking healthy Japanese and in cerebrospinal fluid (CSF) from patients without neurological or inflammatory diseases. The serum NEP activity (sNEP) of 25 males and 25 females, aged 20 to 65 years, ranged from 0.003 to 1.62 pmole/min/ $\mu$ l. There was no significant difference in sNEP activity between the sexes (male:  $0.40 \pm 0.34$  pmole/min/ $\mu$ l vs female:  $0.37 \pm 0.30$ , mean  $\pm$  S.D.). There was a significant positive correlation ( $p < 0.05$ ) between sNEP and age.

The NEP activity in the CSF (cNEP) ranged from 0.07 to 0.63 pmole/min/ $\mu$ l. Male patients with benign prostate hypertrophy (BPH) showed cNEP activity of  $0.21 \pm 0.11$  pmole/min/ $\mu$ l ( $n=13$ ), and female patients with myoma uteri (MU) or dysplasia of the uterus mucosa (DUM) showed activity of  $0.32 \pm 0.20$  ( $n=5$ ). There was no significant difference in cNEP activity between the sexes. Three patients with severe body pain showed cNEP activity of 0.21, 0.15, and 0.16 pmole/min/ $\mu$ l, and these values were not dissimilar from those of the BPH, MU, or DUM patients.

**Key words:** Neutral endopeptidase, Serum, Cerebrospinal fluid, Severe body pain

Neutral endopeptidase (NEP; EC 3.4.24.11) is a membrane-bound ectoenzyme which hydrolyses bioactive peptides including atrial natriuretic peptide (ANP), substance P, and enkephalins<sup>11,13</sup>.

NEP is distributed in many tissues such as kidney, intestine, lung, lymphnodes, and brain<sup>5</sup>. It is also found in a soluble form in plasma, and its activity increases in diseases such as adult respiratory distress syndrome, leukemia, and lung cancer<sup>1,4,6</sup>.

In the brain, NEP is considered to be related to enkephalin degradation, because NEP inhibitors administered to the central nervous system of the mouse showed an analgesic effect<sup>3</sup>.

Recently, the purpose of the soluble form of NEP in the serum has been reported<sup>8</sup>. However, the purpose of the soluble form of NEP in CSF is still unclear.

We measured NEP activity in serum (sNEP) from healthy, non-smoking Japanese, and its age-related variation was investigated. We also measured the NEP activity in CSF (cNEP) from

patients both with and without severe body pain, and compared these values.

### MATERIALS AND METHODS

The following materials were purchased from the companies listed in parentheses: [Leu<sup>5</sup>]Enkephalin, pepstatin A, glycil-glycil-glycine, and phosphoramidon (Protein Research Foundation, Osaka, Japan); bestatin (Nihon Kayaku Co. Ltd., Osaka, Japan); puromycin, and phenylmethylsulphonyl fluoride (Sigma Chemical Co., St. Louis, MO); Hanks balanced salt solution (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan), and HEPES buffer (Katayama Kogyo Co. Ltd., Tokyo, Japan). The captopril was a generous gift from Sankyo Pharmaceutical Co. Ltd., Osaka, Japan. Other reagents were of the special grade.

Serum samples were collected from healthy non-smoking adults (25 males and 25 females), and CSF samples were collected from 13 males with benign prostate hypertrophy (BPH), 3 females with myoma utery (MU), 2 females with dysplasia of the uterus mucosa (DUM), a 61-year-

old female with multiple bone fractures, a 69-year-old male with severe anal pain after surgery, and a 44-year-old male with severe neck pain after a car accident.

Venous blood was drawn from the cubital vein and their sera were kept frozen at  $-30^{\circ}\text{C}$  until the experiment. Hemolysis was carefully avoided during sample collection and the blood samples were completely spun down to avoid contamination by the cell debris to the serum. CSF samples were collected during spinal anesthesia for surgery. The samples were spun down and their supernatants were kept frozen at  $-40^{\circ}\text{C}$  until the experiment. We could not obtain serum samples from these patients.

High performance liquid chromatography (HPLC) was constructed with two pumps, a UV meter, a gradient mixer, a column (TSK gel ODS-120T,  $4.0 \times 120$  mm), an auto sampler (Tosoh Co. Ltd., Tokyo, Japan), and a chromatopac (C-R6A, Shimadzu Co. Ltd., Kyoto, Japan).

The method of measurement of NEP activity has been specified elsewhere<sup>6</sup>, but we modified it in this experiment by adding puromycin as an enzyme inhibitor<sup>12</sup>) and glycil-glycil-glycine as a dipeptidyl peptidase inhibitor. These modifications improved the stability and did not affect NEP activity in the experiment.

Eight microliters of [ $\text{Leu}^5$ ]Enkephalin (0.5 mM in distilled water), 4  $\mu\text{l}$  of bestatin (1 mM in D.W.), 4  $\mu\text{l}$  of Captopril (1 mM in D.W.), 4  $\mu\text{l}$  of pepstatin A (1 mM in dimethyl sulphoxide), 4  $\mu\text{l}$  of phenylmethylsulphonyl fluoride (200 mM in dimethyl sulphoxide), 4  $\mu\text{l}$  of puromycin (1 mM in D.W.) 20  $\mu\text{l}$  of glycil-glycil-glycine (100 mM in D.W.), 8  $\mu\text{l}$  of phosphoramidon (1 mM in D.W.) or D.W., and 319  $\mu\text{l}$  of Hanks balanced salt solution containing 10 mM HEPES buffer (pH 7.4) were mixed together in the tube and preincubated at  $37^{\circ}\text{C}$  for 5 min. Twenty five microliters of the sample serum was then added to the tube, quickly vortexed, and further incubated for 30 min under the same conditions. The reaction was stopped by adding 25% trichloroacetic acid (100  $\mu\text{l}$ ), the proteins were pelleted by centrifugation (Kubota KM-15200, 15,000 rpm, 5 min, at R.T.), and the supernatants were saved.

[ $\text{Leu}^5$ ]Enkephalin hydrolysis was measured by reverse phase HPLC monitoring at OD<sub>280</sub>. Twenty microliter aliquots of the supernatants were injected into the column and eluted with an increasing gradient of acetonitrile (Fig. 1, flow rate 1 ml/min). The residual amount of [ $\text{Leu}^5$ ]Enkephalin was calculated by the integrated peak area of OD<sub>280</sub> and compared to that of a known amount of [ $\text{Leu}^5$ ]Enkephalin.

NEP activity was calculated by the following equation:  $(A-B)/T/V$  (pmole/min/ $\mu\text{l}$ ) where A is the residual [ $\text{Leu}^5$ ]Enkephalin in the tube containing phosphoramidon (pmole), B is the residu-

al [ $\text{Leu}^5$ ]Enkephalin in the tube lacking phosphoramidon (pmole), T is the incubation time (min), and V is the sample volume ( $\mu\text{l}$ ).

Since the [ $\text{Leu}^5$ ]Enkephalin degradation curve was linear up to 30 min and usually around 5% of the total [ $\text{Leu}^5$ ]Enkephalin was degraded during 30-min incubation<sup>6</sup>), we expressed NEP activity as the mean degradation speed of [ $\text{Leu}^5$ ]Enkephalin during 30-min incubation. Each value was expressed as a mean of triplicate measurements.

The statistical analysis of the data was done by the least square method and an unpaired Student's t test.

## RESULTS

The HPLC profile of the sample shows a sharp peak of [ $\text{Leu}^5$ ]Enkephalin (Fig. 1). The coefficient of variation of the three standard [ $\text{Leu}^5$ ]Enkephalin peak areas were 6.28 (10 mM, n=7), 6.94 (25 mM, n=7), and 5.49 (50 mM, n=7).

The serum NEP activity of healthy non-smoking volunteers (25 males and 25 females), aged 20 to 65 years, ranged from 0.003 to 1.62 pmole/min/ $\mu\text{l}$ . There was a positive correlation between sNEP activity and age ( $y=0.19 + 4.18 \times 10^{-3}x$ ,  $p<0.05$ , Fig. 2). We did not find a significant difference in sNEP activity between the sexes

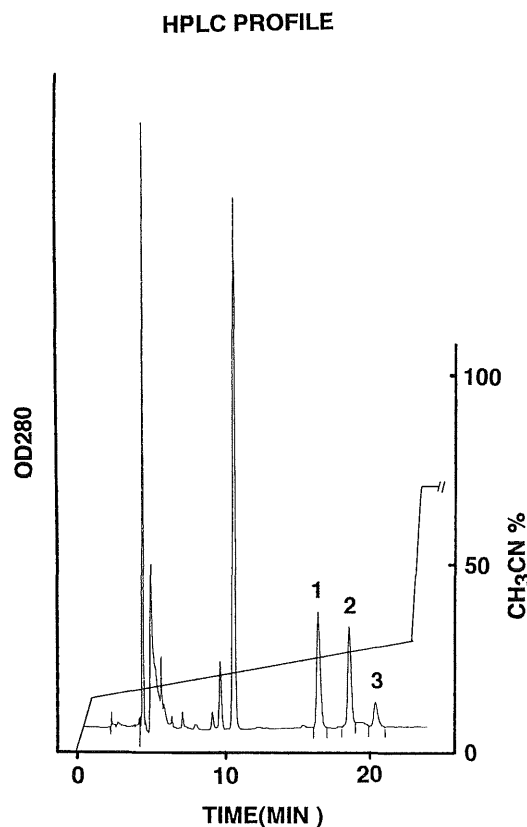
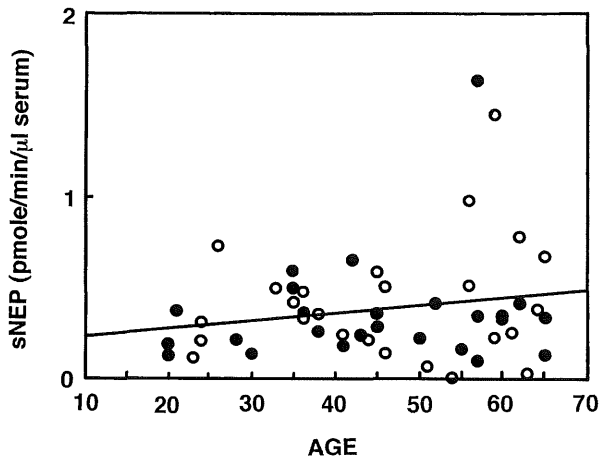


Fig. 1. HPLC profile of the sample. Peak 1 and 2: phosphoramidon and its related peak; peak 3: [ $\text{Leu}^5$ ]Enkephalin



**Fig. 2.** Relationship between sNEP activity and age  
○: male; ●: female

(male:  $0.40 \pm 0.34$  pmole/min/ $\mu$ l vs female:  $0.37 \pm 0.30$ , mean  $\pm$  S.D.).

The cNEP activity of the male patients with BPH was  $0.21 \pm 0.11$  pmole/min/ $\mu$ l ( $n=13$ ), and the activity of the female patients with MU or DUM was  $0.32 \pm 0.20$  ( $n=5$ ) (Table). The differ-

ence in cNEP activity between the sexes was not significant. A 61-year-old female with multiple bone fractures showed cNEP activity of 0.21 pmole/min/ $\mu$ l, a 69-year-old male with severe anal pain after surgery showed 0.15, and a 44-year-old male with severe neck pain after a car accident showed 0.16. These values were not dissimilar from the values of the BPH, MU, or DUM patients.

## DISCUSSION

NEP is a membrane-bound ectoenzyme which is distributed on the cell surface of many organs, including kidney, lymphnodes, intestine, lung, and brain<sup>5</sup>). NEP also exists in the plasma, and its activity is greater in venous blood than in arterial blood, suggesting that the NEP in the plasma comes from the microvessels<sup>10</sup>). Although we do not yet know whether sNEP is a membrane-bound form of NEP which is released from the microvessels or a soluble form of NEP isoenzyme present in the cell, sNEP (as well as NEP in the vascular endothelial cells) affects the concentrations of bioactive peptides in the blood<sup>8</sup>). For example, NEP in the vascular endothelium is able to inactivate ANP and thus, at least in part contributes to the homeostasis of the cardiovascular

**Table** NEP activity in the CSF

name	age(years)	sex	diagnosis	NEP activity (pmole/min/ $\mu$ l)
Y.O.	62	M	BPH	0.23
T.M.	74	M	BPH	0.14
G.T.	74	M	BPH	0.23
S.G.	80	M	BPH	0.22
K.K.	69	M	BPH	0.36
S.F.	74	M	BPH	0.40
K.I.	65	M	BPH	0.12
S.H.	79	M	BPH	0.10
D.I.	63	M	BPH	0.08
M.H.	74	M	BPH	0.33
T.T.	79	M	BPH	0.20
K.M.	66	M	BPH	0.10
T.S.	71	M	BPH	0.26
Y.N.	52	F	MU	0.07
F.H.	73	F	MU	0.24
E.S.	36	F	MU	0.63
K.O.	64	F	DUM	0.34
J.T.	30	F	DUM	0.33

BPH: benign prostate hypertrophy; MU: myoma uteri;  
DUM: dysplasia of the uterus mucosa

system<sup>9</sup>). Although ANP concentration in the serum changes remarkably with age<sup>14</sup>), we do not yet know whether sNEP activity changes with age or not. Another microvessel-derived enzyme, angiotensin converting enzyme (ACE), which also degrades ANP, decreases remarkably in the serum during early life and reaches its adult level before childhood<sup>7</sup>). Our data shows that sNEP also changes with age (Fig. 2), but the pattern of the change is different from ACE.

NEP in the central nervous system may modulate the analgesic effects of opioid peptides, because mice receiving the NEP inhibitors Thiorphan and Bestatin intraventricularly at the same time showed marked analgesic effects and the opioid peptide level rose by more than 50% in one hour<sup>3</sup>). However, we do not know whether cNEP activity is related to pain sensation or not.

Consequently, we measured the cNEP activity of patients both with and without severe body pain. These patients did not have any neurological or inflammatory diseases. Our results show that cNEP levels were comparable with the sNEP levels (Table), and that the cNEP activity of patients with severe body pain did not differ from that of patients without pain. However, it was difficult to evaluate the intensity of pain, and the periods of pain (at most, three weeks) were perhaps not long enough to change the cNEP levels. We could not measure the levels of peptides such as enkephalin and substance P in these samples. Further research is needed to prove the relationship between bioactive peptides and NEP in serum and CSF.

#### ACKNOWLEDGEMENTS

We wish to express our grateful thanks to Mr. M. Hasegawa and Miss M. Seto for their technical assistance.

(Received August 5, 1996)

(Accepted November 12, 1996)

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