Preproenkephalin B-derived opioid peptides in human phaeochromocytomas

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Abstract. We demonstrated the presence and the secretion in vivo and in vitro of immunoreactive preproenkephalin B-derived opioid peptides (\(\alpha\)-neoendorphin, dynorphin and leumorphin) in human phaeochromocytomas. In seventeen human phaeochromocytomas and two human adrenal medullas, the tissue contents of immunoreactive preproenkephalin B-derived opioid peptides (\(\alpha\)-neoendorphin, dynorphin and leumorphin) and leu-enkephalin were studied by specific RIAs. Compared with a remarkable wide distribution in amounts of immunoreactive leu-enkephalin (1063 \pm 437 pg/mg, mean \pm SE), small amounts of immunoreactive \(\alpha\)-neoendorphin (22.6 \pm 6.4 pg/mg) and dynorphin (8.5 \pm 1.2 pg/mg) were detected in all seventeen human phaeochromocytomas and the two human adrenal medullas. Leumorphin-like immunoreactivity was detected in only four tumours. Gel chromatographic studies revealed the presence of preproenkephalin B-derived peptides and their high molecular forms. A significant positive correlation between the tumour tissue contents of immunoreactive \(\alpha\)-neoendorphin and of dynorphin was observed. Nicotine \((10^{-5}, 10^{-4} \text{ mol/l})\) significantly stimulated the secretion of immunoreactive \(\alpha\)-neoendorphin and dynorphin as well as leu-enkephalin and catecholamines from cultured human phaeochromocytoma cells. Administration of 1 mg of glucagon to a patient with medullary phaeochromocytoma induced a rapid increase in the plasma concentration of immunoreactive \(\alpha\)-neoendorphin with a concomitant increase in plasma catecholamines. These results indicate the presence of preproenkephalin B-derived opioid peptides in human phaeochromocytomas and human adrenal medullas and their secretion in human phaeochromocytomas.

Several studies have demonstrated that large amounts of met-enkephalin, its related derivat-
Materials and Methods

Materials

The phaeochromocytomas are listed in Table 1. Phaeochromocytomas obtained at surgery were immediately frozen at −70°C until extraction. Two human adrenal glands were obtained from two women who underwent left adrenalectomy as part of the surgical therapy for breast cancer. Adrenal medullas were carefully dissected out and immediately frozen at −70°C until extraction.

Synthetic peptides, leu-enkephalin and dynorphin (1-13) were obtained from the Protein Research Foundation (Minoh, Osaka, Japan). Antiserum for leu-enkephalin was obtained from Amersham International plc (England). Specific antiserum for dynorphin (1-13) was kindly donated by Dr A. Goldstein (Ghazarossian et al. 1980). Standard α-neoendorphin and specific antiserum for α-neoendorphin (Minamino et al. 1981) were kindly donated by Prof H. Matuso, Miyazaki Medical College, Japan. Standard human leumorphin and specific antiserum for human leumorphin (Nakamura et al. 1985) were kindly donated by Prof Yamahara, Shizuoka College of Pharmacy, Japan.

Tissue extraction

After weighing, tissues were boiled for 10 min in 1 mol/l acetic acid containing 20 mmol/l HCl, homogenized and centrifuged for 15 min at 2500 × g. An aliquot of the supernatant was frozen and lyophilized for later assays of opioid peptides. Because of the acidic nature of leumorphin, the tissues were also extracted in boiled water for RIA of leumorphin according to the method by Nakao et al. (1983).

Column study

Lyophilized tissue extract was reconstituted in 1 mol/l acetic acid and applied on a 1 × 47 cm Sephadex column G-50 (Pharmacia) equilibrated with 1 mol/l acetic acid. The elution was performed with 1 mol/l acetic acid. The fraction volume of 1 ml was frozen and lyophilized for later assays of opioid peptides.

Cell culture

Isolation of phaeochromocytoma cells from a patient (No. 2) was performed following the method previously described (Yanase et al. 1984). Finally, human phaeochromocytoma cells were plated in plastic dishes (Falcon 3001). Each dish contained 1 × 10⁶ cells in 1 ml of Iscove’s Modified Eagles Medium (IMDM) (GIBCO) supplemented with 10% foetal calf serum (GIBCO). The cells were incubated at 37°C in 5% CO₂-95% air. The media were daily renewed and the experiments were initiated 3 days after plating. Before the addition of nicotine, each plate was washed at 37°C for 5 min in 1.0 ml of Hepes-buffered Krebs-Ringer saline (KRH) containing 0.2% bovine serum albumin (BSA) and then exchanged with 1.0 ml of KRH containing 2 mmol/l CaCl₂ and 5 × 10⁻⁵ mol/l Bacitracin (Sigma). Thereafter, 10⁻⁵ or 10⁻⁴ mol/l nicotine was added to each plate. After 15 min of incubation, 200 µl of the media was acidified with 50 µl of 2 mol/l perchloric acid for subsequent estimation of catecholamines and the remaining 800 µl was acidified with 800 µl of 2 mol/l acetic acid and lyophilized for subsequent estimation of opioid peptides. These experiments were performed in triplicate dishes.

Glucagon administration

Glucagon (1 mg, Nova Industri, Bagsværd, Denmark) was injected iv into patient No. 1 and blood samples were taken at 0, 3 and 15 min after the injection. All blood samples were drawn into chilled, siliconized glass tubes containing EDTA 2Na and centrifuged at 4°C. The plasma samples were frozen until extraction.

Plasma extraction

Two ml of the plasma samples for RIA of opioid peptides were subjected to Sep-Pak cartridge (Waters Associates). After applying each sample, the cartridge was rinsed with 4% acetic acid and adsorbed extracts were eluted with methanol. These eluates were evaporated under nitrogen gas stream. The extraction rate of α-neoendorphin was calculated by the recoveries of 125I-labelled peptide added in plasma through extraction. Recoveries of 125I-α-neoendorphin were 81.0 ± 4.8% (mean ± SD). With 2 ml of extraction volume, the lower detection limit of α-neoendorphin in plasma was 7.5 ng/l, after correction by extraction rate.

Catecholamine determination

Tissue contents of catecholamines were determined by high-pressure liquid chromatography (HPLC) (Hitachi 635A) after purification by alumina (Anton & Sayre 1962). Catecholamines in the cultured medium were directly measured by HPLC. The samples were applied to the column (Hitachi gel 3011-C, 0.4 × 15 cm), eluted with 0.1 mol/l phosphate buffer (pH 2.9, flow rate 0.6 ml/min), and measured by a fluorescence method (Ueda et al. 1977). The sensitivity of the assay was 10 ng/l. Plasma catecholamines following glucagon administration were measured by HPLC in collaboration with SRL (Special Reference Laboratory, Tokyo). Normal ranges of plasma epinephrine and norepinephrine were below 0.12 and 0.06–0.45 µg/l, respectively.

RIA

Radioimnations were performed by the chloramine T method (Miller et al. 1978) The 125I-labelled peptides were purified on CM-cellulose (CM52) column or Sephadex G-10, 25 column. The RIA buffer used was 0.05 mol/l phosphate buffer (pH 7.4) containing 0.25% (wt/vol) BSA, 0.08 mol/l NaCl, 0.025 mol/l EDTA-2Na, and 0.05% (wt/vol) NaN₃. The cross-reactivity (mol per mol) of the respective antiserum for dynorphin, α-neo-
endorphin, and leumorphin with other opioid peptides are precisely described in the respective original papers (Ghazarossian et al. 1980; Minamino et al. 1981; Nakamura et al. 1985). In summary, an antiserum for leu-enkephalin cross-reacted 5% with met-enkephalin and less than 0.1% with other peptides, including \( \alpha \)-neoendorphin, dynorphin (1-13), and human leumorphin. The respective antiserums for \( \alpha \)-neoendorphin, dynorphin (1-13), and human leumorphin showed no significant cross-reactivity with each other or with leu-enkephalin (< 0.1%). An antiserum for dynorphin (1-13) cross-reacted 100% with complete sequence of dynorphin (1-17) isolated as endogenous proline pituitary dynorphin (Goldstein et al. 1981). An antiserum for human leumorphin mainly recognized the C-terminal peptides of human leumorphin and cross-reacted 78% with human leumorphin (15-29), however it showed no cross with rimorphin (leumorphin 1-13) isolated from posterior pituitary glands (Kilpatrick et al. 1982). The sensitivities of these RIAs were 3–6 pg/tube.

**Statistics**
Statistical analysis was performed using Student’s \( t \)-test.

**Results**
The tumour contents of catecholamines and immunoreactive leu-enkephalin, \( \alpha \)-neoendorphin, dynorphin, and human leumorphin are shown in Table 1. The dilution curves of tissue extracts were parallel with the standard curves of the respective RIAs for opioid peptides. A remarkable wide distribution of leu-enkephalin (18–5482 pg/mg tissue, 1065 ± 437 pg/mg, mean ± se) was demonstrated among the tumours. The tissue contents of immunoreactive \( \alpha \)-neoendorphin (22.6 ± 6.4 pg/ml) and dynorphin (8.5 ± 1.2 pg/mg) were depicted in all tissues, however leumorphin-like immunoreactivities were detected only in four tumours (Nos. 1, 2, 3 and 6) in water extracts and only in one tumour (No. 2) in acid extracts. A significant positive correlation between the tumour tissue contents of immunoreactive \( \alpha \)-neoendorphin and dynorphin was observed \(( \gamma = 0.804, P < 0.01)\). No significant correlation

**Table 1.**
List of phaeochromocytomas and tissue contents of catecholamines and immunoreactive leu-enkephalin, \( \alpha \)-neoendorphin, dynorphin and leumorphin.

<table>
<thead>
<tr>
<th>Tumour No.</th>
<th>Tumour weight (g)</th>
<th>Patient Age/Sex</th>
<th>Epinephrine (pg/mg)</th>
<th>Norepinephrine (pg/mg)</th>
<th>Leu-enkephalin (pg/mg)</th>
<th>( \alpha )-neoendorphin (pg/mg)</th>
<th>Dynorphin (pg/mg)</th>
<th>Leumorphin (pg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (M)</td>
<td>25</td>
<td>24/F</td>
<td>11.82</td>
<td>8.21</td>
<td>5482</td>
<td>69.6</td>
<td>13.9</td>
<td>ND (2.5)</td>
</tr>
<tr>
<td>2 (M)</td>
<td>11</td>
<td>61/M</td>
<td>3.43</td>
<td>2.40</td>
<td>4899</td>
<td>101.3</td>
<td>20.8</td>
<td>5.4 (8.2)</td>
</tr>
<tr>
<td>3 (M)</td>
<td>80</td>
<td>42/F</td>
<td>3.30</td>
<td>2.40</td>
<td>2500</td>
<td>27.8</td>
<td>8.2</td>
<td>ND (1.1)</td>
</tr>
<tr>
<td>4 (M)</td>
<td>260</td>
<td>59/F</td>
<td>0.01</td>
<td>1.19</td>
<td>965</td>
<td>10.2</td>
<td>7.6</td>
<td>ND</td>
</tr>
<tr>
<td>5 (M)</td>
<td>75</td>
<td>16/M</td>
<td>0.32</td>
<td>12.81</td>
<td>100</td>
<td>2.5</td>
<td>6.0</td>
<td>ND</td>
</tr>
<tr>
<td>6 (M)</td>
<td>26</td>
<td>42/M</td>
<td>4.56</td>
<td>0.50</td>
<td>3134</td>
<td>31.2</td>
<td>12.8</td>
<td>ND (1.7)</td>
</tr>
<tr>
<td>7 (M)</td>
<td>272</td>
<td>46/M</td>
<td>0.18</td>
<td>3.01</td>
<td>298</td>
<td>8.4</td>
<td>3.2</td>
<td>ND</td>
</tr>
<tr>
<td>8 (M)</td>
<td>202</td>
<td>34/F</td>
<td>0.12</td>
<td>3.87</td>
<td>329</td>
<td>5.7</td>
<td>4.3</td>
<td>ND</td>
</tr>
<tr>
<td>9 (ExM)</td>
<td>13</td>
<td>35/F</td>
<td>ND</td>
<td>2.43</td>
<td>49</td>
<td>18.2</td>
<td>10.6</td>
<td>ND</td>
</tr>
<tr>
<td>10 (ExM)</td>
<td>11</td>
<td>ND</td>
<td>ND</td>
<td>1.86</td>
<td>23</td>
<td>10.8</td>
<td>4.9</td>
<td>ND</td>
</tr>
<tr>
<td>11 (ExM)</td>
<td>14</td>
<td>37/F*</td>
<td>ND</td>
<td>2.34</td>
<td>56</td>
<td>37.0</td>
<td>9.8</td>
<td>ND</td>
</tr>
<tr>
<td>12 (ExM)</td>
<td>18</td>
<td>ND</td>
<td>ND</td>
<td>2.04</td>
<td>60</td>
<td>11.1</td>
<td>13.6</td>
<td>ND</td>
</tr>
<tr>
<td>13 (ExM)</td>
<td>12</td>
<td>ND</td>
<td>ND</td>
<td>1.64</td>
<td>75</td>
<td>14.0</td>
<td>6.8</td>
<td>ND</td>
</tr>
<tr>
<td>14 (ExM)</td>
<td>450</td>
<td>45/F</td>
<td>ND</td>
<td>0.19</td>
<td>40</td>
<td>2.0</td>
<td>1.5</td>
<td>ND</td>
</tr>
<tr>
<td>15 (ExM)</td>
<td>38</td>
<td>24/M</td>
<td>ND</td>
<td>1.06</td>
<td>24</td>
<td>24.0</td>
<td>8.8</td>
<td>ND</td>
</tr>
<tr>
<td>16 (ExM)</td>
<td>45</td>
<td>ND</td>
<td>ND</td>
<td>0.57</td>
<td>18</td>
<td>6.4</td>
<td>8.2</td>
<td>ND</td>
</tr>
<tr>
<td>17 (ExM)</td>
<td>10</td>
<td>ND</td>
<td>ND</td>
<td>0.89</td>
<td>18</td>
<td>4.9</td>
<td>4.3</td>
<td>ND</td>
</tr>
<tr>
<td>Human adrenal medulla</td>
<td>1</td>
<td>7.2</td>
<td>3.6</td>
<td>534</td>
<td>18.8</td>
<td>14.1</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5.6</td>
<td>3.0</td>
<td>220</td>
<td>34.8</td>
<td>22.2</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

Bracketed values represent leumorphin-like immunoreactivity by water extraction of the tissue. ND: not detectable both by acidic extraction and water extraction. M and ExM represent medullary and extramedullary, respectively. 37/F* represents a recurrent case of 35/F.
Gel filtration profiles of immunoreactive α-neoendorphin, dynorphin and leumorphin on Sephadex G-50 column (1 x 47 cm) of extract of two human phaeochromocytomas (Nos. 2 and 4) and a human adrenal medulla. I : 125I-α-neoendorphin, II : 125I-dynorphin (1-13), III : 125I-leumorphin.

was demonstrable between tumour tissue contents of immunoreactive leu-enkephalin and dynorphin/α-neoendorphin. There were no significant differences as to tissue contents of immunoreactive α-neoendorphin and dynorphin between medullary phaeochromocytomas and extramedullary phaeochromocytomas. Gel chromatographic studies of preproenkephalin B-derived peptides on a Sephadex G-50 column of extracts from two tumours and human adrenal medulla are shown in Fig. 1. Immunoreactive α-neoendorphin consisted of a peak emerging at the position of synthetic α-neoendorphin and a peak at the position of high molecular weight. Immunoreactive dynorphin consisted of a peak emerging a little forward to the position of synthetic dynorphin (1-13) implying dynorphin (1-17) and one at the high molecular weight position. Immunoreactive leumorphin consisted of a peak emerging at the position of synthetic leumorphin and a peak of probable C-terminal fragments of leumorphin.

**Secretion experiments in culture**

Secretion experiments with nicotine from cul-

**Fig. 1.**

**Fig. 2.**

Effect of nicotine on the secretion of catecholamines and opioid peptides from cultured human phaeochromocytoma cells obtained from a tumour in patient 2.

*P < 0.05 vs control.
tured human phaeochromocytoma cells (No. 2) are shown in Fig. 2. Addition of $10^{-5}$ and $10^{-4}$ mol/l nicotine significantly stimulated the secretion of not only catecholamines and leu-enkephalin but also α-neoendorphin and dynorphin.

**Glucagon administration**

Basal plasma concentration of immunoreactive α-neoendorphin was not detected. Basal plasma epinephrine and norepinephrine were within normal range. Glucagon, 1 mg, iv, resulted in a rapid increase in plasma catecholamines and α-neoendorphin in a patient (No. 1) with medullary phaeochromocytoma only three min after the injection. Fifteen min after the injection, plasma levels of α-neoendorphin were still higher than the basal level, whereas plasma epinephrine and norepinephrine returned to normal range (Table 2). During the test, the blood pressure increased from 90/42 to 160/90 mmHg after 3 min and returned to 82/35 mmHg after 15 min.

**Discussion**

Preproenkephalin B-derived peptides have not been clearly elucidated in human adrenal medullas and phaeochromocytomas other than by a few reports showing the presence of minor dynorphin-like immunoreactivity in these tissues (Yoshimasa et al. 1981; Suda et al. 1983) and a report showing the absence of B-derived peptides in the human adrenal medulla (Evans et al. 1984). In the present study, we demonstrated the presence of α-neoendorphin and dynorphin, preproenkephalin B-derived peptides, in both human phaeochromocytomas and adrenal medullas by use of specific RIA’s. In addition, we obtained first evidence of the co-secretion of immunoreactive preproenkephalin B-derived opioid peptides and catecholamines both in phaeochromocytoma cell culture with nicotine and following glucagon administration in a patient with medullary phaeochromocytoma. These results indicate the synthesis and the processing of the preproenkephalin B molecule in human phaeochromocytomas.

Although leumorphin has already been proven to be an endogeneous opioid peptide in the central nervous system and gastrointestinal tract (Nakamura et al. 1985), it was not detected in most phaeochromocytomas and human adrenal medullas probably because of the absence of processing. The question arises whether leu-enkephalin could result from the processing of α-neoendorphin and dynorphin, α-neoendorphin and dynorphin containing leu-enkephalin and the successive dibasic amino acids Arg-Lys and Arg-Arg, respectively, in the N-terminal. From our results yielding no significant correlation between leu-enkephalin and α-neoendorphin/dynorphin, preproenkephalin B seems to be less responsible for the production of leu-enkephalin than preproenkephalin A.

We could confirm the increase in plasma immunoreactivity of α-neoendorphin following glucagon administration in a patient with medullary phaeochromocytoma. Concomitant increases in the immunoreactivity of met-enkephalin and of leu-enkephalin in plasma following glucagon administration was reported by Yoshimasa et al. (1983). Preproenkephalin B-derived peptides are secreted from human phaeochromocytomas and may have some physiological roles in peripheral tissues, including the inhibitory effect on catecholamine secretion from human Ph cells (Yanase et al. 1986).

In summary, we showed the presence of preproenkephalin B-derived opioid peptides in human phaeochromocytomas and human adrenal medullas and their secretion in human phaeochromocytomas.

**Acknowledgments**

We would like to acknowledge the generous collaboration of the Department of Urology (Director: Prof J. Kumazawa), Kyushu University. This study was supported in part by Grant-in-Aid for Special Project Research of Selected Intractable Neurological Disorders, the Ministry of Education, and by a research grant from the Research Committee of Adrenal Hormone Disorders, the Ministry of Health and Welfare, Japan.
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Received June 1st, 1986.
Accepted November 14th, 1986.
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