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Effect of *Epilobium angustifolium* L. extracts and polyphenols on cell proliferation and neutral endopeptidase activity in selected cell lines

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The ability of *Epilobium* extracts and polyphenols to induce neutral endopeptidase (NEP) activity and to inhibit the proliferation in cell lines with high NEP expression (SK-N-SH) and with low NEP expression (PC-3) was investigated. *Epilobium* extracts enhanced in a dose-depend manner NEP activity in both cell lines with additional inhibition of cell proliferation. The sensitivity of cells depended on basal enzyme activity. SK-N-SK cells were much more sensitive than PC-3 cells. Oenothein B enhanced NEP activity at a concentration of 5–40 μM while quercetin-3-glucuronide and quercetin-3-*O*-(6″-galloyl) galactoside showed slight or no activity at a concentration of 100 μM. The comparison of activities of the extracts with oenothein B, a dimeric macrocyclic ellagitannin, suggests that the latter is mostly responsible for the observed effects. Taking into account the role of NEP in the homeostasis of signalling peptides, *Epilobium angustifolium* extracts may be a potential herbal remedy in diseases connected with the disturbed metabolism of signaling peptides caused by an unbalanced neutral endopeptidase activity.

1. Introduction

Neutral endopeptidase (NEP; EC 3.4.24.11), a zinc metallopeptidase located on the outer membrane of different cells, catalyses the degradation of a variety of renal and CNS-active peptides, including substance P, bradykinin, enkephalins and atrial natriuretic peptides (Turner et al. 2001). In the prostate gland NEP deactivates bombesin, endothelin-1 and calcitonin-gene-related peptide. As a result, the loss of NEP activity or expression may promote peptide-mediated proliferation, suggesting a crucial role of NEP in various cancers. Decrease of NEP expression has been reported in renal and bladder cancers (Gohring et al. 1998; Koiso et al. 1994), endometrial cancer (Ino et al. 2004), small cell and non-small cell lung cancers (Cohen et al. 1996). Recent research has revealed that neutral endopeptidase is involved in the transition from hormonally regulated androgen-dependent prostate cancer to androgen-independent prostate cancer (Papandreou et al. 1998). In several studies of the expression of NEP in androgen-dependent (LNCaP) and androgen-independent (DU 145 and PC-3) cell lines, high levels of neutral endopeptidase mRNA and protein expression have been found for LNCaP cells but not for both androgen-independent cell lines.

The inhibition of NEP by phosphoramidon increases the proliferation of neuroblastoma cells treated with a vasoactive intestinal peptide which plays a role in the up-regulation of tumor angiogenesis (Wollman et al. 2002). Additionally, brain NEP can degrade neurotoxic amyloid peptide involved in Alzheimer's disease (Iwata et al. 2001).

Taking into account the role of NEP in the homeostasis of signalling peptides, we investigated the influence of *Epilobium* extracts and *Epilobium*-derived polyphenols on NEP activity and proliferation in two different cell lines. We chose cells with high NEP expression (SK-N-SH) and with low NEP expression (PC-3). The anti-proliferative activity of *Epilobium* extracts on prostatic epithelial (PZ-HPV-7), LNCaP, astrocytoma (1321N1) and mammary epithelial (HMEC) cell lines has been demonstrated (Vitalone et al. 2003) but no study concerning NEP activity in cell lines has been carried out till now.

2. Investigations and results

2.1. SK-N-SH

Table 1 shows the effect of long-term incubation of the SK-N-SH cells with methanolic (ME) and aqueous (AE) extracts of *Epilobium* in concentrations of 25–100 μg/ml. Treatment resulted in a dose-dependent enhancement of NEP activity compared with the basal activity (7.1 ± 0.8 pmolAMC/min per μg of DNA, $n = 30$). The treatment with both extracts led to a significant inhibition of cell proliferation. These results indicate that the up-regulation of NEP activity is connected with a decrease in cellular proliferation. Between tested polyphenols, oenothein B (OeB) strongly induced NEP activity at a rather low concentration (5–20 μM), while quercetin-3-glucuronide (QG) and quercetin-3-*O*-(6″-galloyl) galactoside (QGG) showed slight or no activity (Table 2). The ellagitannin also strongly inhibited cell proliferation. The

Table 1: Influence of *Epilobium* extracts on SK-N-SK and PC-3 specific enzyme activity and cell proliferation

Treatment	Specific cellular NEP activity in %	Cell proliferation %
SK-N-SH		
Control	100 ± 5	100 ± 6
ME 25 µg/ml	142 ± 16*	70 ± 5**
ME 50 µg/ml	193 ± 21*	59 ± 10**
ME 100 µg/ml	919 ± 156*	13 ± 3**
AE 25 µg/ml	139 ± 17*	76 ± 10**
AE 50 µg/ml	309 ± 89*	35 ± 6**
AE 100 µg/ml	2679 ± 208*	4 ± 1**
PC-3		
Control	100 ± 7	100 ± 6
ME 25 µg/ml	110 ± 13	100 ± 11
ME 50 µg/ml	130 ± 17*	90 ± 6
ME 100 µg/ml	148 ± 19*	82 ± 5**
AE 25 µg/ml	138 ± 10*	84 ± 6**
AE 50 µg/ml	150 ± 12*	80 ± 6**
AE 100 µg/ml	166 ± 12*	74 ± 4**

* Significant difference to the specific enzymatic activity of the controls
** Significant difference to cell proliferation of the controls

Table 2: Influence of *Epilobium* polyphenols on SK-N-SK and PC-3 specific enzyme activity and cell proliferation

Treatment	Specific cellular NEP activity in %	Cell proliferation %
SK-N-SH		
Control	100 ± 11	100 ± 10
OeB 5 µM	159 ± 9*	56 ± 6**
OeB 10 µM	257 ± 36*	35 ± 5**
OeB 20 µM	1072 ± 73*	9 ± 1**
QG 25 µM	117 ± 15	85 ± 4
QG 50 µM	137 ± 10*	67 ± 5**
QG 100 µM	159 ± 12*	55 ± 11**
QGG 25 µM	118 ± 26	91 ± 6
QGG 50 µM	111 ± 21	84 ± 16
QGG 100 µM	111 ± 5	71 ± 13**
PC-3		
Control	100 ± 6	100 ± 4
OeB 10 µM	131 ± 10*	93 ± 6
OeB 20 µM	153 ± 15*	83 ± 4**
OeB 40 µM	219 ± 20*	69 ± 3**
QG 100 µM	96 ± 4	90 ± 3
QGG 100 µM	93 ± 11	90 ± 4

* Significant difference to the specific enzymatic activity of the controls
** Significant difference to cell proliferation of the controls

comparison of activities of the extracts with oenothein B suggests that the latter is mostly responsible for the observed effects.

2.2. PC-3

Table 1 and 2 show that long-term treatment of PC-3 cells with aqueous extract (25–100 µg/ml) and oenothein B (10–40 µM) resulted in a dose-dependent enhancement of NEP activity compared with the basal activity (0.61 ± 0.06 pmolAMC/min per µg of DNA, n = 30). In addition, the AE and the ellagitannin both showed a weak but statistically significant inhibition of cell proliferation. A methanolic extract showed a weaker effect, while QG and QGG were inactive even in a concentration of 100 µM. As in a SK-N-SK cell, the up-regulation of NEP activity by the extract is probably due to the presence of oenothein B.

3. Discussion

In our investigation we demonstrated that *Epilobium* extracts enhanced in a dose-dependent manner the activity of NEP in both cell lines with an additional inhibition of cell proliferation. The sensitivity of cells depended on the basal enzyme activity. SK-N-SK cells were much more sensitive than PC-3 cells and the concentration of 100 µg/ml of both extracts was toxic for a neuroblastoma cell. In order to find compound(s) responsible for observed activity of the extracts we tested three polyphenols from *Epilobium angustifolium* which in our previous study influenced the activity of isolated enzymes (Kiss et al. 2004). Oenothein B, a dimeric macrocyclic ellagitannin, was the most active compound. The presence of this ellagitannin appears to be responsible for the activity of the extracts. An aqueous extract was more active than an alcoholic one, probably due to a better water solubility of tannin.

Two flavonoids showed weaker activity. However, quercetin glucuronid was able to inhibit the proliferation of SK-N-SK cell with statistically significant induction of NEP activity. The aglycon quercetin (2.5 µM) itself has been shown to inhibit strongly the proliferation of SK-N-SK cell and to enhance the activity of metallopeptidases (Melzig and Escher 2002). The growth of PC-3 cell was also inhibited by quercetin but at 10 fold higher concentration (Knowles et al. 2000). Interestingly, *in vitro* QG is able to cross the small intestine and blood brain barriers, and reach the CNS (Juergenliemk et al. 2003).

The possible explanations of the observed induction of NEP activity might be based on the fact that the up-regulation of NEP cellular enzymatic activity in different cell types was correlated with the inhibition of cellular proliferation and expressed in an enhanced differentiation state. During the induction of BeWo cell differentiation by forskolin, a time-depend enhancement of NEP expression and activity was observed (Uehara et al. 2001). Yang et al. (1994) found out that TNF-α can induce a differentiation of the neuroblastoma SK-N-SH cell to perineurium-like cells. OeB has been shown to have antitumor activity, probably due to the stimulation of the release of interleukin IL-1β and tumor necrosis factor (TNF-α) (Miyamoto et al. 1993; Feldman et al. 1999). In contrast TNF-α had no effect on PC-3 cell line (Nakijima et al. 1995), suggesting that the activity of extracts and OeB, at least in prostate cancer cells, is not due to the release of TNF-α. In our previous study, we demonstrated that *Epilobium* extracts and OeB inhibit NEP activity used an isolated enzyme (Kiss et al. 2004). Perhaps, in a more physiological environment as it is in a living cell, the ellagitannin may induce expression and activity of NEP mediated by binding at the ectoenzyme connected with intracellular signalling via the intracellular domain of the enzyme.

The possible physiological importance of the described effects of *Epilobium* extracts should be discussed in relation to the physiological role of NEP in different tissues. The increase in cellular NEP activity might be associated with an increased neuropeptide metabolism which is necessary to maintain the functional integrity of different tissues and organs. Bombesin, endothelin-1 and calcitonin-gene-related peptide which are involved in proliferation of prostatic cells and are degraded by NEP may be presumably involved in the pathogenesis of prostate diseases. Recent research has revealed that changes in neutral endopeptidase expression are implicated in the progression of prostate cancer (Papandreou et al. 1998). Freedland et al. (2003) demonstrated that patients with prostatic intraepithelial neo-

plasia (PIN) showed an intermediate level of NEP expression between prostate cancer and normal prostatic tissue, suggesting that a loss of NEP may occur at an early premalignant phase and that NEP may be a useful therapeutic target for early stage disease. Recombinant NEP has been shown to promote anticancer drug-induced apoptosis in PC-3 cell and to augment chemosensitivity with diminished toxicity in normal tissues (Sumitomo et al. 2004).
Another aspect is the bioavailability of OeB. Information about the metabolism of ellagitannins is scarce. However, it has been reported that mouse sera taken after oral administration of OeB inhibited the replication of HIV and herpes virus, suggesting that this ellagitannin may be bioavailable (Okuda et al. 1989).
In summary, our results demonstrate that *Epilobium angustifolium* extracts may serve as a potential herbal remedy in diseases connected with a disturbed metabolism of signaling peptides caused by an unbalanced neutral endopeptidase activity. Other studies mainly concerning the bioavailability of oenothein B are needed to demonstrate the possible clinical efficacy of this herbal drug.

4. Experimental

4.1. Materials

Suc-L-Ala-L-Ala-Phe-7-amino-4-methyl-coumarin (SAAP-AMC), phosphoramidon, bisbenzimidazole (Hoechst 33258) and aminopeptidase N were purchased from Sigma. Cell culture media and foetal bovine serum (FBS) were obtained from Biochrom. Oenothein B (OeB), quercetin-3-glucuronide (QG) and quercetin-3-*O*-(6″-galloyl) galactoside (QGG) were isolated as described previously (Kiss et al. 2004). The structure and purity was confirmed by ^{1}H and ^{13}C NMR.

4.2. Extract preparation

The herb of *Epilobium angustifolium* L. was collected in the north-east Poland and identified by Prof. B. Sudnik-Wójcikowska of the Department of Environmental Botany, Warsaw University. The herb was dried at room temperature. A specimen of the drug (No 02031) is available in the drug collection of the Department of Pharmacognosy, Medical University, Warsaw (Poland).
70% Methanolic extract (ME): 50g of powdered plant material was extracted with 70% methanol (1 : 10) in an ultrasonic water bath for 1 h at 40 °C. After methanol evaporation, the water residue was lyophilised.
Aqueous extract (AE): 50 g of powdered plant material was extracted with water (1 : 10) in an ultrasonic water bath for 1 h at 40 °C. After filtration the filtrate was lyophilised.

4.3. Cell culture

4.3.1. SK-N-SH cells

SK-N-SH cells, human neuroblastoma cells, were obtained from ATCC (No. HTB-11) and cultivated in Minimal Essential Medium (MEM with Earls salts), supplemented with sodium pyruvat and non-essential amino acids plus 10% FBS, at 37 °C in a humidified atmosphere containing 5% CO_2. Subcultivation was performed in 70 cm^2 culture flasks until confluence. For the enzymatic experiments, cells were seeded in 24-well plates (inoculum 100.000 cells/well). 24 h after plating, the cells were incubated with the indicated concentrations of test compound dissolved in buffer saline and cultivated for a further 4 days. The medium was then removed and replaced by NEP-assay solution.

4.3.2. PC-3 cells

PC-3 cells, human androgen-independent prostate cancer cells, were obtained from PD Dr. Kleuser (FU Berlin) and cultivated in RPMI 1640 medium, supplemented with 10% FBS, at 37 °C in a humidified atmosphere containing 5% CO_2. Subcultivation was performed in 150 cm^2 culture flasks until confluence. For the enzymatic experiments, cells were seeded in 24-well plates (inoculum 100.000 cells/well). 24 h after plating, the cells were incubated with the indicated concentrations of test compound dissolved in buffer saline and cultivated for a further 4 days. The medium was then removed and replaced by NEP-assay solution.

4.4. Determination of NEP activity

A two-step assay, according to Bormann and Melzig (2000) was performed. 50 µl of SAAP-AMC (400 µM) and 400 µl of HEPES-buffer (50 mM + 154 mM NaCl, pH 7.4) were added to the intact cell layer and incubated for 60 min at 37 °C. The reaction was stopped by the addition of 50 µl of phosphoramidon solution (50 µM). 400 µl of the incubation mixture from each well were transferred in an Eppendorf tube. 20 µl of APN-solution (1 : 235) were added, and the reaction mixture was incubated again for 60 min at 56 °C. The reaction was terminated by the addition of 800 µl of acetone. The fluorescence of the released AMC was measured at $\lambda_{exit} = 367$ nm and $\lambda_{emiss} = 440$ nm. A calibration curve with AMC was used to calculate the enzyme activity.
The enzyme activity was calculated in pmol/min per µg of DNA.

4.5. Cell proliferation

Cell proliferation was determined by cellular DNA content in 24-well plates after enzyme assay. DNA content was determined using the fluorochrome bisbenzimidazole (Hoechst 33258) after cell lysis by freezing with water (Rago et al. 1990). Briefly, after enzyme activity determination, cells were washed with buffer saline, and 400 µl of water were then added. Plates were frozen twice, 100 µl of each well were transferred in an Eppendorf tube, and 640 µl of phosphate-saline buffer (50 mM $NaPO_4 = 2$ M NaCl + 2 mM EDTA, pH = 7.4) and 60 µl of Hoechst solution (10 µg/ml in water) were added. Fluorescence was measured at $\lambda_{exit} = 356$ nm and $\lambda_{emiss} = 458$ nm. A calibration curve with herring testes DNA (Sigma) was used to calculate the DNA amount per well.

4.6. Statistics

All results were presented as a mean ± standard error of the mean (SEM) of at least three independent experiments (each performed with 4–6 parallel samples). Statistical analysis was performed using the student's t-test, with $p < 0.05$.

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