Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical Pharmacology 69 (2005) 1441–1450
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Representative aminopeptidases and prolyl endopeptidase from murine macrophages: Comparative activity levels in resident and elicited cells

Renata do Amaral Olivo, Catarina de Fátima Pereira Teixeira, Paulo Flávio Silveira *

[a]Laboratory of Pharmacology, Instituto Butantan, Av. Dr.Vital Brazil, 1500-CEP, 05503-900 São Paulo, SP, Brazil

Received 14 October 2004; accepted 4 March 2005

Abstract

Macrophages are considered the main effector cells of immune system. Under stimulation these cells are known to be activated by a process involving morphological, biochemical and functional changes. Since altered peptidase activities could be among the factors leading to the differentiation and activation of these cells, in the present work seven naphthylamide derivative substrates were employed to assess representative aminopeptidase and prolyl endopeptidase activities in resident and elicited macrophages of mice. Soluble basic aminopeptidase and prolyl endopeptidase and soluble and particulate neutral and prolyl dipeptidyl aminopeptidase IV activities were present at measurable levels while particulate prolyl endopeptidase and basic aminopeptidase, and particulate and soluble cystyl and pyroglutamyl aminopeptidases were not detectable. Kinetic parameters, chloride activation and the inhibitory effects of puromycin, bestatin, amastatin and diprotin A characterized differential properties of these peptidase activities. The observed increment (about 6–17-fold) of the soluble basic aminopeptidase and prolyl endopeptidase and soluble and particulate neutral and prolyl dipeptidyl aminopeptidase IV activities in elicited macrophages was particularly relevant, as these might contribute to an increased ability of this cell to inactivate several susceptible substrates known to be inflammatory and/or immunological mediators.

Keywords: Macrophages; Peptidases; Peptides; Mediators; Inflammation; Immune system

1. Introduction

Macrophages are differentiated end cells with the ability to recognize and destroy altered host components such as apoptotic cells and invading organisms. They play a central role in immunological and inflammatory processes in physiological and pathological conditions [1]. These cells are found in several organs and tissues such as serous cavities. Resident macrophages are among the first lines of host defense since these physiologically quiescenT-cells become activated by a number of external stimuli of varying origin [2]. Activated macrophages express low secretory activity but present a high capacity in secreting reactive-oxygen or -nitrogen species, and important microbicidal and tumoricidal activities [3,4]. In addition, inflammatory or immunological stimuli evoke a recruitment of blood circulating monocytes to specific sites, where they differentiate into elicited macrophages. These elicited cells present a high secretory activity and phagocytic capacity but a low microbicidal and tumoricidal activity [5,6]. All these selected activities from distinct populations of macrophages modulate the reaction of tissues to external stimuli.

Factors leading to the differentiation and activation of macrophages remain largely uncharacterized. Aminopeptidase (AP) was among the earliest peptidase activity proposed as a marker of macrophage activation [7,8]. In spite of the wide diversity of peptide substrates related to inflammatory and immune responses, limited information is available regarding the induction of peptidases in leukocytes. Membrane and soluble proteinases in macrophages have been linked with integrins and other adhesion proteins and with intracellular signaling systems underlying cell migration [9]. Recently the interest in these enzymes in leukocytes has increased considerably, since several reports have indicated the roles of these peptidases and related peptide substrates in several physiopathological processes in many tissues [10–16]. Furthermore, among very few endopeptidases that are able to cleave the prolyl

* Corresponding author. Tel.: +55 11 3726 7222.
E-mail address: pefesil@butantan.gov.br (P.F. Silveira).

0006-2952/$ – see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2005.03.002

bond, prolyl endopeptidase is the unique which has been reported to be present in murine macrophages [17]. In the light of actual knowledge, it is believed that peptidases may have a role in macrophage function not only through hydrolytic action on peptide mediators, resulting in activation or inhibition, but also as receptors and mediators of adhesion or transduction signals. However, kinetics and simultaneous evaluation of representative aminopeptidase and prolyl endopeptidase activities have not yet been characterized in macrophages. Moreover, the activities of these peptidases in distinct stages of stimulation are still unclear.

To learn more about the peptidase activities of murine macrophages we investigated the activities of soluble (S) and membrane-bound (M) acid (APA), basic (APB), neutral (APN), cystyl (CAP), pyroglutamyl (PAP), prolyl dipeptidyl IV (DPPIV) aminopeptidases and prolyl endopeptidase (PEP) in resident (RE) and thioglycollate-elicited (TGE) isolated cells. Here we show that certain peptidase activities were absent or present at negligible levels in resident and elicited macrophages, while those detectable in residenT-cells also increased in elicited cells.

2. Material and methods

2.1. Animals

Male Swiss mice were maintained in a restricted-access room with a controlled temperature of 25 °C, relative humidity of 65.3 ± 0.9% and 12 h light:12 h dark photoperiod (lights on at 6:00 h a.m.), and were housed in cages (inside length × width × height of 56 cm × 35 cm × 19 cm), with a maximum of 20 mice per cage, with food and tap water ad libitum. All animals weighed 18–20 g.

The animal and research protocols used in this study are in agreement with the Brazilian Council Directive (COBEA – BRAZIL) and were approved by the Ethics Committee of the Instituto Butantan (035/2001).

2.2. Obtention of resident (RE) and thioglycollate-elicited (TGE) macrophages

Resident macrophages were harvested by washing peritoneal cavities. Briefly, animals were killed under halotane and exsanguined. Then, peritoneal lavage was performed, after a gentle massage of the abdominal wall. The peritoneal fluid, containing resident macrophages, was collected. Aliquots of the washes were used to determine total peritoneal cells numbers in a Neubauer's chamber after dilution (1:20, v/v) in Turk solution. The predominance of mononuclear cells in the washes was confirmed by light microscopic analysis of smears stained with Hema [3]. The cell population consisted of around 99% macrophage as determined by morphological criteria. Washes were then centrifuged at 200 × *g*, 6 min, 22°, and the pellet obtained re-suspended in 2.0 mL of 10 mM Tris-HCl, pH 7.4.

Thioglycollate-elicited macrophages were harvested 4 days after intraperitoneal (i.p.) injection of 1.0 mL of 3% thioglycollate and peritoneal lavage was performed according to the above description. The cell population consisted of more than 95% macrophage as determined by morphological criteria.

2.3. Protein

Protein was measured at 630 nm in triplicate by the Bio-Rad protein assay [18] using a Bio-Tek FL600FA Microplate Fluorescence/Absorbance Reader. Protein content was extrapolated by comparison with a standard curve of bovine serum albumin (BSA) (Sigma, St. Louis, MO, USA) diluted in 10 mM Tris-maleate buffer, pH 5.9.

2.4. Preparation of the soluble (S) and solubilized membrane-bound (M) fractions from macrophages

Macrophage suspensions in 10 mM Tris-HCl buffer, pH 7.4 were sonicated at room temperature at amplitude 40 for 10 s and ultracentrifuged (Hitachi model HIMAC CP60E) at 100,000 × *g* for 35 min. The resulting supernatants were used to measure the S enzyme activities and protein concentrations. To avoid contamination with the S, the resulting pellet was washed three times with 10 mM Tris-HCl buffer, pH 7.4. The pellet was then homogenized for 30 s at 800 rpm in 10 mM Tris-HCl buffer, pH 7.4, plus 0.1% Triton X-100 and ultracentrifuged at 100,000 × *g* for 35 min. The supernatants thus obtained were used to determine the M enzyme activities and protein concentrations. All steps were carried out at 4 °C.

2.5. Lactate dehydrogenase (LDH)

As a marker for the fractionation procedure, LDH activity was determined spectrophotometrically at 340 nm with a Molecular Devices®-Spectra Max 190 [19] in the S and M fractions. 30 μL samples of S or M were incubated with 270 μL of 100 mM phosphate buffer, pH 7.4, containing 200 mM NaCl and 1.6 mM sodium pyruvate solution (Sigma, St. Louis, MO, USA) plus 0.2 mM nicotinamide adenine dinucleotide, reduced form (NADH) disodium salt (Sigma, St. Louis, MO, USA). Values of LDH activity were obtained by the results of subtraction of the absorbance at 340 nm read at 10 min from that read at 0 time of incubation at 37 °C and extrapolated by comparison with a standard curve of NADH. LDH activity was expressed as mmol NADH oxidized/min/mg protein.

2.6. Peptidase assays

Enzyme activities were quantified on the basis of the amount of 4-methoxy-β-naphthylamine (Sigma) (for

DPPIV) or β-naphthylamine (Sigma) (for all other peptidases) released as a result of incubation at 37 °C for 30 min in 96-well flat botton microplates (Corning Inc., NY, USA) of 50-μL samples and 250-μL pre-warmed substrate solution in such respective 0.05 M buffers containing BSA 0.1 mg/mL. β-Naphthylamine or 4-methoxy-β-naphthylamine, were estimated fluorometrically with a Bio-Tek FL600FA Microplate Fluorescence/Absorbance Reader, at 460/40 nm emission wavelength and 360/40 nm excitation wavelength in triplicate samples. The value for the incubates at zero time (blank) was subtracted and the relative fluorescence was converted to picomoles of β-naphthylamine or 4-methoxy-β-naphthylamine by comparison with a correspondent standard curve. APA activity was measured as described by Ramírez-Expósito et al. [20] using L-aspartic acid α-(β-naphthylamide) (Sigma) in Tris-HCl buffer, pH 7.4, with 1mM $MnCl_2$. CAP activity was measured as described by Silveira et al. [21] using L-cystine di-β-naphthylamide (Sigma) in Tris-maleate, pH 5.9. APN and APB activities were measured as described by Mantle [22] respectively using L-alanine-β-naphthylamide in phosphate buffer, pH 7.4, with 1 mM DL-dithiothreitol (DTT) (Sigma), or L-arginine-β-naphthylamide (Sigma) in phosphate buffer, pH 6.5, with 150 mM NaCl, and 0.02 mM puromycin. PAP activity was measured by the method of Schwabe and McDonald [23] using L-pyroglutamic acid-β-naphthylamide (Sigma) in phosphate buffer, pH 7.4, with 2 mM DTT, and 2 mM ethylenediaminetetraacetic acid (EDTA) (Merck, Brazil). DPPIV activity was measured by the method of Liu and Hansen [24] using H-Gly-Pro-4-methoxy-β-naphthylamide (Peninsula, USA) in Tris-HCl buffer, pH 8.3. PEP activity was measured by the method of Zolfaghari et al. [25], using Z-Gly-Pro-β-naphthylamide (Bachem, USA) in phosphate buffer, pH 7.4, with 2 mM DTT.

2.7. Data analysis

Data were analyzed statistically using GraphPad Prism® and Instat® softwares. Regression analyses were performed to obtain standard curves for protein concentration and enzyme activity measurements. Two-tailed Student's *t*-test was performed to compare the values of resident and elicited macrophages from the same enzyme activity and the effect of NaCl on APB or PEP activities. One-way analysis of variance (ANOVA) was performed to detect differences in all parameters in such macrophage status, followed by the Tukey–Kramer multiple comparisons test when differences were detected. Differences were considered statistically significant at $P < 0.05$.

3. Results

Table 1 shows that LDH activity was 7–26-fold higher in S than in M fractions, which confirms the efficiency of the adopted fractionation procedure.

Table 1
Lactate dehydrogenase (LDH) activity in soluble (S) and membrane-bound (M) fractions of resident (RE) and thioglycollate-elicited (TGE) macrophages from mice

Macrophage status	LDH activity	
	S	M
RE	0.79	0.03
TGE	0.45	0.06

LDH activity expressed as mmol NADH oxidized/min/mg protein. Values are means ± S.E.M from five animals. Values on the same line were measurements in triplicate for the same animals. All paired values in different columns were significantly different (two-tailed paired Student's *t*-test, $P < 0.001$).

The M forms of APB and PEP, and S and M forms of APA, CAP and PAP activities were not detectable while Table 2 shows that S-APB and S-PEP and S and M APN and DPPIV were detected at considerable levels in macrophages. As also shown in Table 2, Michaelis constant (K_m), turnover number (K_{cat}) and the specificity constant (K_{cat}/K_m) were calculated for such activities after the incubation of the macrophage suspension containing 4.3×10^6 cells/mL with different concentration ranges of naphthylamide derivative substrates (APN and PEP: 3.125–125 μM; APB: 3.125–250 μM; DPPIV: 5–200 μM) at 37 °C for 30 min. Further measurements of S and M peptidase activities of RE and TGE macrophages shown in Figs. 1–4 are the results of sample incubation with substrate solutions diluted to 0.5 mM (for APB) or 0.2 mM (for DPPIV) or 0.125 mM (for APN and PEP) in such respective buffers. Enzyme activity was expressed as picomoles of substrate hydrolyzed per minute (UP) per milligram of protein. Assays were linear with respect to time of hydrolysis, protein content, and number of cells.

Table 3 shows the effects of selected inhibitors on peptidase activity of TGE macrophages. At the employed concentrations, distinct inhibitory potency observed on such enzyme activities can be summarized as follows: (i) puromycin: S-APN = M-APN = APB = PEP > S-DPPIV > M-DPPIV; (ii) bestatin: APB = PEP = M-APN > S-APN > S-DPPIV = M-DPPIV; (iii) amastatin: APB = PEP = S-APN = M-APN > S-DPPIV = M-DPPIV; (iv) diprotin A: S-APN = M-APN < APB = PEP = S-DPPIV = M-DPPIV. Regarding the susceptibilities of such enzyme activities to each one of these inhibitors, Fig. 1 shows the following: (i) S-DPPIV: diprotin A > puromycin > amastatin > bestatin; (ii) M-DPPIV: diprotin A > puromycin > amastatin > bestatin; (iii) S-APN: amastatin > diprotin A; M-APN: diprotin A < puromycin = bestatin = amastatin. APB and PEP presented the same susceptibility to all these inhibitors. As shown in Fig. 2, APB was activated while PEP was not affected by the presence of NaCl.

As shown in Fig. 3, it is noteworthy that peptidase activities (UP/mg protein; values are means ± S.E.M; $n = 5$) strongly increased in activated macrophages from 244.6 ± 16.7 (RE) to 1342.6 ± 267.2 (TGE) for S-APB,

Table 2
Kinetic parameters of basic (APB), neutral (APN), prolyl dipeptidyl IV (DPPIV) aminopeptidase and prolyl endopeptidase (PEP) activities obtained by fluorometric measurements of hydrolysis of naphthylamide derivative substrates by soluble (S) and membrane-bound (M) fractions from a pool of thioglycollate-elicited macrophages from mice

Substrate β-naphthylamide	Enzyme	V_{max} (pmoles min^{-1} mg $protein^{-1}$)	K_{cat} (s^{-1})	$K_m \times 10^{-5}$ (M)	$K_{cat}/K_m \times 10^{-5}$ (M^{-1} s^{-1})
Arg-	S-APB	$3455 \pm 461^{*}$	57.6^{*}	$18.2 \pm 3.3^{*}$	3.16
Ala-	S-APN	$810 \pm 17^{*}$	13.5^{*}	1.9 ± 0.1	7.11^{*}
Ala-	M-APN	227 ± 60	3.78	1.2 ± 0.7	3.15
H-Gly-Pro-4-methoxy-	S-DPPIV	195 ± 46	3.25	3.2 ± 1.2	1.01
H-Gly-Pro-4-methoxy-	M-DPPIV	300 ± 31	5	1.8 ± 0.3	2.78
Z-Gly-Pro-	S-PEP	284 ± 18	4.7	1.4 ± 0.2	3.36

Values are mean ± S.E.M. of three assays with a pool of macrophages obtained from thirty mice 96 h after intraperitoneal injection of 3% thioglycollate. Values for K_{cat} [maximum amount of substrate (picomoles) converted per second per enzyme unit (UI)] were calculated considering 1 UI = amount of enzyme, in 1 mg of protein, which hydrolyses 1 picomol of substrate per second. Kinetic analysis was performed by Eadie–Hofstee methodology (slope = K_m; y intercept = V_{max}), using GraphPad Prism® software. * $P < 0.05$ in comparison to other values in the same column (ANOVA, Tukey–Kramer multiple comparisons test).

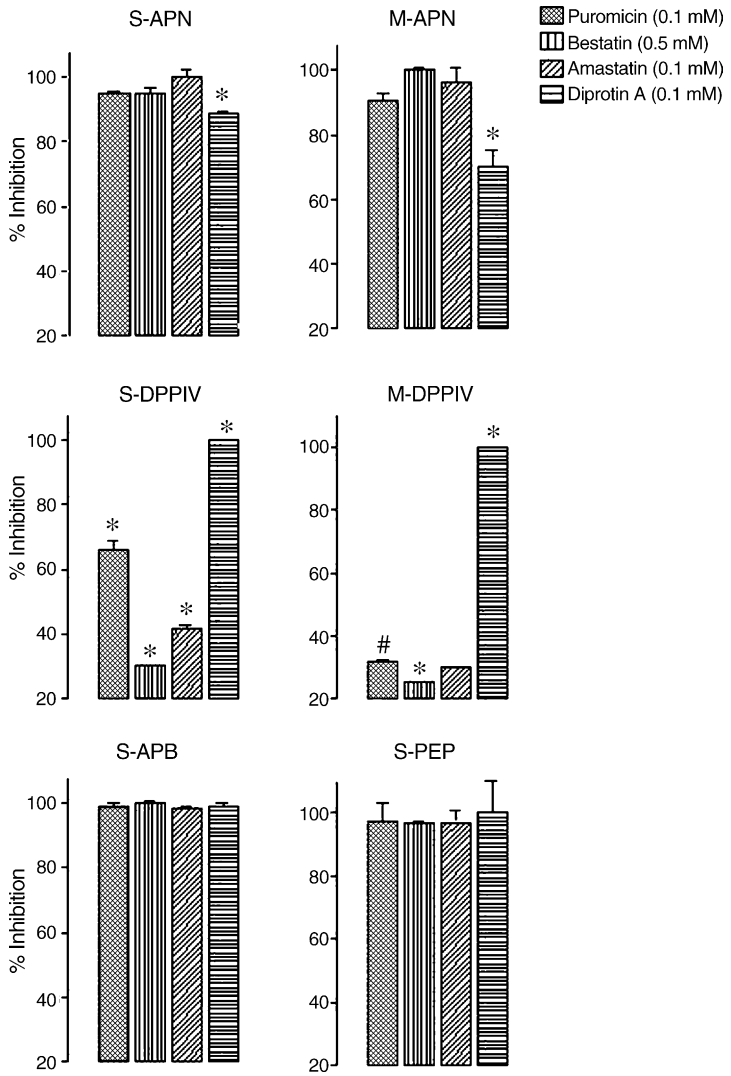

Fig. 1. Enzyme susceptibilities to selected inhibitors of basic (APB), neutral (APN), prolyl dipeptidyl IV (DPPIV) aminopeptidase and prolyl endopeptidase (PEP) activities in soluble (S) and membrane-bound (M) fractions from thioglycollate-elicited macrophages from mice. Values are mean ± S.E.M. from a pool of 10 animals. Percentage of inhibition was calculated considering the absence of inhibitors as 100% of activity. * $P < 0.05$ in comparison to other values of same enzyme activity; $^{\#}$ $P < 0.05$ in comparison to Diprotin A effect on the same enzyme activity (ANOVA, Tukey–Kramer multiple comparisons test). See Table 3 for comparison of susceptibilities of distinct enzyme activities to the same inhibitor.

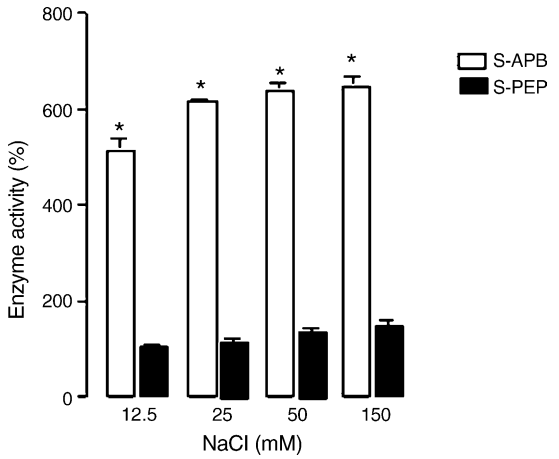

Fig. 2. Effect of NaCl on soluble (S) basic aminopeptidase (APB) and prolyl endopeptidase (PEP) activities from thioglycollate-elicited macrophages. Values are mean ± S.E.M. from a pool of 10 animals. Percentage of activity was calculated considering the absence of NaCl as 100% of activity. * $P < 0.05$ in comparison to the absence of NaCl (two-tailed paired Student's *t*-test).

from 25.4 ± 6.1 (RE) to 158.3 ± 18.3 (TGE) for S-APN, from 6.2 ± 2 (RE) to 46.9 ± 9.7 (TGE) for M-APN, from 51 ± 8 (RE) to 550.2 ± 48.7 (TGE) for S-DPPIV, from 2.9 ± 0.6 (RE) to 50.9 ± 12.4 (TGE) for M-DPPIV, and from 18 ± 3 (RE) to 177.1 ± 37.7 (TGE) for PEP. These increments represent proportional changes in the relative percentage of all examined enzyme activities in these different macrophage status with the exception of DPPIV which was increased in S and M fractions of TGE in relation to RE (Table 4). In both macrophage status, the sum of the relative distribution of all enzyme activities was higher in S than in M forms with S-APB as the most prominent activity (Fig. 4).

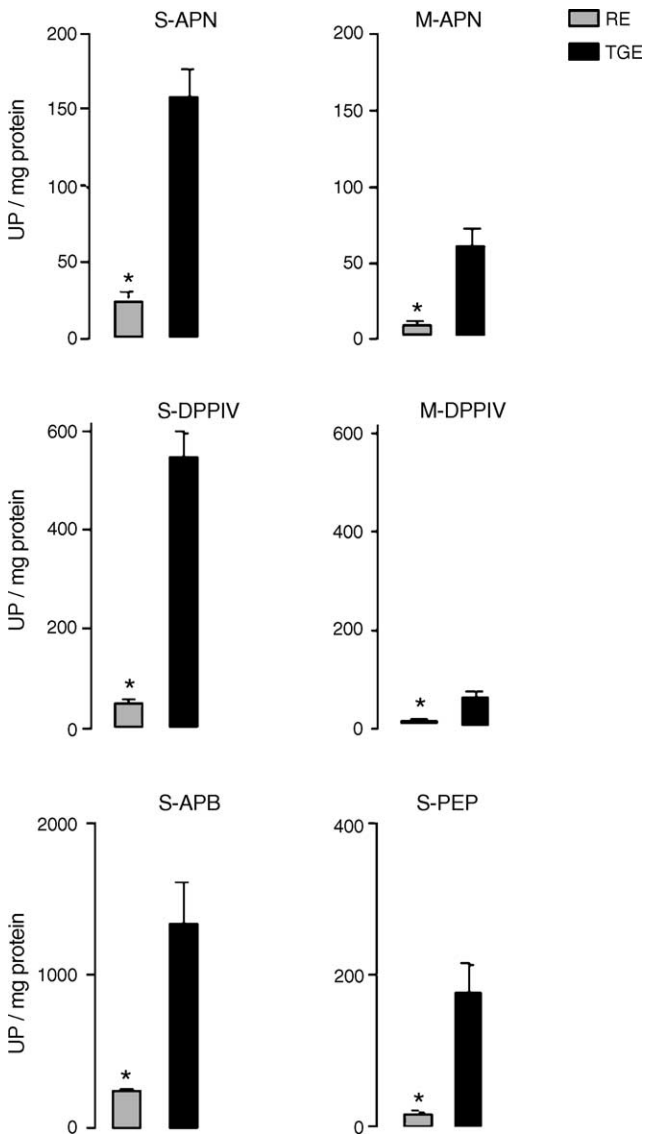

Fig. 3. Soluble and membrane-bound peptidase activities (UP/mg protein) of resident (RE) and thioglycollate-elicited (TGE) macrophages from mice. Values are means ± S.E.M from five animals (assays made in triplicate). * $P < 0.05$ in comparison to the elicited macrophages for the same enzyme activity (two-tailed unpaired Student's *t*-test). See in Fig. 4 the distribution of the peptidase activity percentages in a single macrophage status and in Table 4 the comparison of peptidase activity percentages between resident and elicited macrophages.

4. Discussion

The present results document the measurements of prolyl endopeptidase and representative AP activities by the assay methods based on the well-characterized differential properties of the hydrolysis of β-naphthylamide or methoxy-β-naphthylamide with different aminoacyl residues in the presence of agents such as puromycin, bestatin, amastatin, diprotin, DTT, EDTA, $MnCl_2$, and NaCl, which are known to produce the activation or inhibition of the enzyme activities under study. The use of naphthylamide derivatives has been the primary step for detection or confirmation of the involvement of AP and prolyl endopeptidase activities in various physiological processes. To our knowledge, this is the first report on the concomitant evaluation of the hydrolytic ability of S and M fractions obtained from resident and elicited murine macrophages on seven different β-naphthylamide derivative substrates which pointed out that S-PEP, S-APB, S-APN, M-APN, S-DPPIV and M-DPPIV change their activities in elicited cells. On the other hand, our results showed that M-PEP, M-APB, and M and S forms of APA, CAP and PAP activities were absent or present at negligible levels in both resident and elicited murine macrophages.

Peptidase activity levels reported here might not be taken as the enzyme activity of one protein on only one substrate. Several data exist that different substrate hydrolysis measured by the use of xenobiotic aminoacyl derivatives do not necessarily represent the specific activity of different proteins. In this regard, puromycin-sensitive and -insensitive neutral aminopeptidases cleave alanyl β-naphthylamide [26,27] and three distinct forms of pyroglutamyl peptidase, comprising a mammalian omega

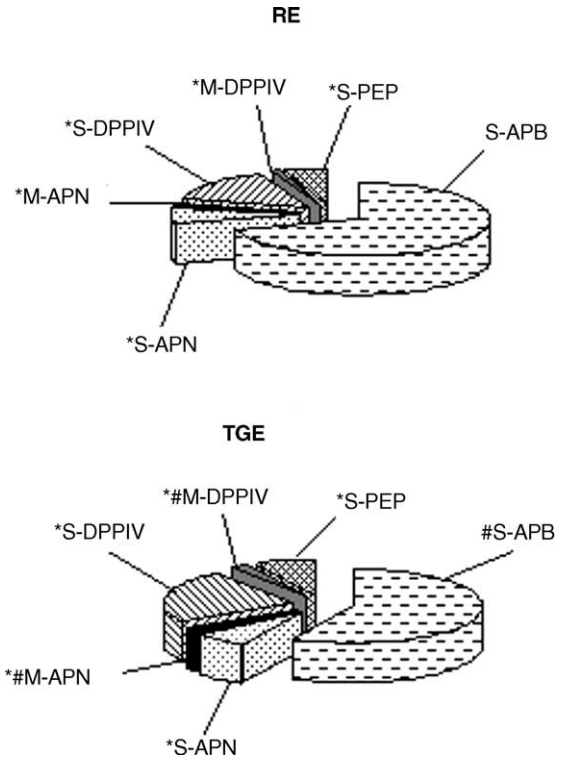

Fig. 4. Distribution of soluble (S) and membrane-bound (M) basic (APB), neutral (APN) and prolyl dipeptidyl IV (DPPIV) aminopeptidase and prolyl endopeptidase (PEP) activities in resident (RE) and thioglycollate-elicited (TGE) macrophages from mice. Sections represent the relative percentage of enzyme activities which were calculated considering the sum of all these activities in the same macrophage status as 100% (see Table 4). * $P < 0.05$ in comparison to S-APB in the same macrophage status; $^{\#}$ $P < 0.05$ in comparison to S-DPPIV in the same macrophage status (ANOVA, Tukey–Kramer multiple comparisons test). See in Table 4 the comparison of peptidase activity percentages between resident and elicited macrophages.

peptidase class, have pyroglutamyl β-naphthylamidase activity [28]. Moreover, a neuronal alanyl aminopeptidase activity exhibits distinct biochemical features from all other known aminopeptidases such as inhibitor specificity, hydrolytic coefficiency, metal effects, p*I*, molecular weight, and catalytic site [29]. In addition, the sensitivity of an alanyl aminopeptidase activity to inhibitors in human T-cell lymphoma line (Jurkat) is markedly increased after disrupting the cells, but still differs from that observed with purified pig neutral aminopeptidase [30]. Similarly, in human liver cytosol, a purified aminopeptidase, which is strongly inhibited by bestatin, leuhistin, actinonin, amastatin and puromycin, has superimposed abilities to hydrolyse the substrates Ala-, Lys-, Phe-, Met-, Leu-, Tyr- and Lys-Ala- 4-methyl-7-coumarylamides at pH ranging from 7.5 to 8.0 [31]. Finally, two distinct molecules (140 and 260-kDa) with alanyl aminopeptidase activity suffer a cross-linking reaction in serum from cholestatic patients [32].

Estimated kinetic parameters and inhibition profiles highlight some distinct features of the peptidase activities under study. They provide background for the optimization of the assay conditions and further biochemical purification of the proteins related to these enzyme activities. In addition, based on the parameters obtained, the differences in the expression and regulation of these enzymes, and the receptors responsible for the observed induction, can be further investigated. We confirmed here the occurrence of certain selectivity with the studied inhibitors on AP activities. However, we could not associate only one inhibitor to only one peptidase activity at least in the employed concentrations. Furthermore, we detected the effect of these so called ''aminopeptidase inhibitors'' on PEP activity.

The affinity of APB was lower (higher K_m) than those of all other measured peptidases. K_{cat} of APB was about 4–10-fold higher in relation respectively to S-APN or to all other measured peptidase activities. However, this relatively high K_{cat} of APB was not followed by an increment of catalytic efficiency (K_{cat}/K_m). S-APN was the activity which presented the highest relative value of K_{cat}/K_m, while from a percentual quantitative viewpoint, S-APB was the most prominent activity when compared with all other macrophage peptidase activities. The aminopeptidase activities under study are known to be sensitive to puromycin, bestatin, amastatin and diprotin A at equal or higher concentrations than those employed in the present study [33–38]. Thus, our inhibitory trials demonstrated that puromycin allowed for the distinction of DPPIV activity between S and M fractions and also this activity in both fractions from those of all the other examined peptidase activities. Bestatin permited to distinguish APN activity between S and M fractions and also between this activity in both fractions from DPPIV activity of S or M fractions.

Table 3
Effects of selected inhibitors on basic (APB), neutral (APN), prolyl dipeptidyl IV (DPPIV) aminopeptidase and prolyl endopeptidase (PEP) activities in soluble (S) and membrane-bound (M) fractions from thioglycollate-elicited macrophages from mice

Inhibitors	Inhibition of peptidase activities (%)					
	S-APN	M-APN	S-DPPIV	M-DPPIV	S-APB	S-PEP
Puromycin 0.1 mM	95.3 ± 0.003	90.7 ± 2.3	66 ± 2.8*	32 ± 0.2*	98.8 ± 1.3	97.1 ± 7
Bestatin 0.5 mM	94.8 ± 1.9*	100 ± 0.8	30.3 ± 0.1$^{\#}$	25.5 ± 0.007$^{\#}$	100 ± 0.6	97 ± 0.09
Amastatin 0.1 mM	100 ± 2.3	96 ± 5	41.4 ± 1.6$^{\#}$	30.3 ± 0.1$^{\#}$	98.5 ± 0.4	96.9 ± 3.9
Diprotin A 0.1 mM	88.9 ± 0.18	70.1 ± 5.3**	100 ± 0.3	100 ± 0.03	99 ± 0.1	100 ± 10

Values are mean ± S.E.M. from a pool of 10 animals. Percentage of inhibition was calculated considering the absence of inhibitors as 100% of activity. * $P < 0.05$ in comparison to other values in the same line; ** $P < 0.05$ in comparison to S-DPPIV, M-DPPIV, S-APB and S-PEP; $^{\#}$ $P < 0.05$ in comparison to S-APN, M-APN, S-APB and S-PEP (ANOVA, Tukey–Kramer multiple comparisons test). See Fig. 1 for comparison of effects of selected inhibitors on the same enzyme activity.

Table 4
Relative activities of basic (APB), neutral (APN), prolyl dipeptidyl IV (DPPIV) aminopeptidase and prolyl endopeptidase (PEP) from soluble (S) and membrane-bound (M) fractions of resident (RE) and thioglycollate-elicited (TGE) macrophages from mice

Macrophage status	Enzyme activities (%)					
	S-APN	M-APN	S-DPPIV	M-DPPIV	S-APB	S-PEP
RE	7.3 ± 1.8	1.8 ± 0.6	14.7 ± 2.3	0.8 ± 0.5	70.2 ± 5	5.2 ± 0.9
TGE	6.8 ± 0.8	2 ± 0.4	23.7 ± 2.1*	2.2 ± 0.5*	57.7 ± 11	7.6 ± 1.6

Percentages were calculated considering the sum of the enzyme activities in the same macrophage status as 100%. Values are mean ± S.E.M. from five animals (assays made in triplicate). * $P < 0.05$ in comparison to resident macrophages (two-tailed unpaired Student's *t*-test). See in Fig. 4 the distribution of the peptidase activity percentages in a single macrophage status.

Bestatin also permited to distinguish between DPPIV activity in S or M fractions from APB and PEP activities. Amastatin permited to distinguish DPPIV activity of S or M from those of all other examined peptidase activities. Diprotin A did not exhibit distinctive inhibitory effect on S or M APN activity but permitted to distinguish this activity of M fraction from all other examined peptidase activities. Diprotin A was also the most potent inhibitor for S or M DPPIV activity while it was the lowest potent for M-APN. Regarding S-APN the inhibitory effect of diprotin A only differed from that of amastatin. Lastly, all these assayed inhibitors had undistinguishable effects on APB and PEP activities. At this point the use of Z-Pro-prolinal could not add relevant information, since a PEP activity resistant to Z-Pro-prolinal inhibition has been described [39] and thus, this activity would still be considered a PEP activity. However, our results showing activation of APB by NaCl clearly differentiated it from PEP. This permitted us to reach our objective of characterizing differential properties of all these examined peptidase activities in macrophages. Chloride-activated APB has also been found in cytosolic fractions of human erythrocytes, polymorphonuclears, monocytes and lymphocytes [40]. Our results show that although only APN and DPPIV presented detectable activity in particulate fraction, even so their relative percentual activities tend to be higher in S than in M forms. Thus our findings strongly suggest that the studied peptidase activities of murine resident and elicited macrophages can be considered as predominantly directed towards peptides arising from the intracellular breakdown of functional proteins, as proposed for T lymphocytes [41], and/or peptides excised from endocytosed peptide receptor complexes, as seems to occur in the human cerebral cortex [42]. However, in addition to the particulate forms, these soluble enzymes could be exocytosed and then could also act on released peptides [43]. The expression of peptidases by leukocytes and other cells with a prominent regulated secretory pathway had already been found to be determined largely by stimulus-response secretion of proteins prepackaged in high concentration. The regulated secretory pathway has seemed minor in macrophages, and instead, peptidases are either channeled into lysosomes or secreted constitutively. Post-translational regulation of macrophage peptidases then seems to depend on compartmentalizing enzymes to their sites of primary function, which have been reported to occur by means of both specific receptors and inhibitors [44]. Another possibility to be considered is that peptides formed by these enzymes could be exocytosed.

Our results showing the increment of S-PEP, S-APB, S-APN, M-APN, S-DPPIV and M-DPPIV activities in elicited macrophages may indicate an enhanced cleavage of peptides related to this cell function. Despite this increment, the relative percentual distribution of S-PEP, S-APB, S-APN and M-APN remained unaltered in both of the studied status of cells. Altered enzyme activities observed in the present study might be predictive of their functional role in macrophages.

In the case of DPPIV we observed that the relative percentage of distribution was increased in S and M fractions of thioglycollate-elicited macrophages in relation to residenT-cells. This finding suggests that differential pathways of stimulation could lead to the increased expression of DPPIV enzyme(s) in macrophages. Upregulation of DPPIV/CD26 mediated by interferons (IFN) was described in chronic B lymphocytic leukemia cells [45]. DPPIV activity removes dipeptides from the aminoterminus of peptides containing proline as the penultimate amino acid [46]. This enzyme activity inactivates inflammatory mediators such as substance P [47], neuropeptide Y [48], and macrophage derived chemokines IL-4, IL-13 and IFN [15]. This enzyme operates in the cascade of immune responses. Independent of its catalytic activity, DPPIV (CD-26) participates in T-cell activation, and binds adenosine deaminase to the T-cell surface, thus protecting them from the inhibition of proliferation [12,49]. Despite the actual role of the increased DPPIV activity in thioglycollate-elicited macrophages had not been identified in the present study, our data point out that the induction of this enzyme activity can profoundly alter the macrophage response. To our knowledge, this is the first demonstration that elicited-cells have increased levels of DPPIV activity. Our suggestive data that DPPIV have a crucial role in the protective function of macrophages deserves further investigation.

APN has been shown to cleave the inflammatory mediators interleukin-8 (IL-8) and neurokinin A [14,50]. Membrane-bound APN/CD-13 is a marker of peripheral blood mononuclear cells [16]. This activity has also been reported to participate in trimming peptides bound to MHC class II molecules [51]. Indeed, the expression of

MHC-II and I molecules for antigen presentation to T-cells is an important function of macrophages. The cleavage of MIP-1 chemokine by APN/CD13 originates in a chemotatic product for eosinophils [52]. Therefore, the increased APN activity may have a role in the increased host defense functions in elicited macrophages. Lymphocytes has been reported to exhibit a rapid induction (dependent on cell-to-cell contact) not only in the CD13 protein but also in Ala-*p*-nitroanilide-cleaving enzyme activity (APN activity) and in CD13 mRNA [53]. APN/CD13 has found to be upregulated by lymphokines such as IFN-γ and IL-4 [54,55]. The increased expression of APN has been related to the reduction of apoptotic neutrophils [56].

APB activity is known to remove arginyl and lysyl residues from the N terminus of small peptides such as the thymopentin. In leukocytes, this activity has been described to be present together with leukotriene A_4 hydrolase activity (LTA_4H) [10,15], which is a key activity in the biosynthesis of the proinflammatory leukotriene B_4 [10]. The induction of the neutrophil LTA_4H activity has been reported to be concomitant with an inhibition of the APB activity of this dual function enzyme, resulting in sustained effects of proinflammatory peptides such as dynorphin fragment 1–7 [57]. Thus, increased activity of APB in elicited macrophages may counterpart the activation of the inflammatory activity of these cells.

PEP activity is known as post-proline cleaving enzyme activity, TRH-deaminase activity or kininase B activity. It cleaves Pro-Xaa bonds in peptides that consist of an acyl-Yaa-Pro-Xaa sequence as found in bradykinin and substance P [58,59].

While much more remain to be seen concerning the pathways leading to the upregulation of S-APB, S-PEP, S-APN, S-DPPIV, M-APN and M-DPPIV or whether this regulation contributes to macrophage differentiation, our data indicate that the increment of these peptidase activities might increase the cellular ability to inactivate several inflammatory and immunological mediators. It is conceivable that altered levels of these peptidase activities affect the processing of peptides bound in the groove of MHC molecules or the properties of receptors and mediators of adhesion or transduction signals. Alternatively, altered levels of these peptidase activities could directly stimulate or activate the macrophages. Moreover, comparison of these enzyme sensitivities to the studied inhibitors in a whole material from macrophages, which are key elements in inflammation and immunology, permits to include these inhibitors among those with potential ability to interfere with inflammatory and immune diseases. Literature data provide strong evidences that members of the AP and PEP families are relevant targets for drug design. The inhibitors of these enzymes seem to be promising drug candidates to treat and prevent certain diseases [60–64].

In conclusion, our data show the concomitant presence of the activities of S-APB, S-PEP, S-APN, S-DPPIV, M-APN and M-DPPIV and suggest that observed increment of these activities in elicited cells might contribute to the downregulation of peptide-mediated function of murine macrophages.

Acknowledgements

This investigation was supported by research grants 00/10023-8 and 02/13863-2 from FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo – Brazil) to P.F.S and C.F.P.T, respectively. P.F.S. and C.F.P.T are the recipients of CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico - Brazil) productivity grants. R.A.O is the recipient of a masters degree fellowship from FAPESP (grant 02/10861-9).

References

[1] Stoy N. Macrophage biology and pathobiology in the evolution of immune responses: A functional analysis. Pathobiology 2001;69:179–211.
[2] Calder PC. Fuel utilization by cells of the immune system. Proc Nutrition Soc 1995;34:65–82.
[3] Cleveland RP, Meltzer MS, Zbar B. Tumor cytotoxicity in vitro by macrophages from mice infected with Mycobacterium bovis strain BCG. Infect Immun 1974;52:1993–8.
[4] Karnovsky ML, Lazdins JK. Biochemical criteria for activated macrophages. J Immunol 1978;121:809–13.
[5] Baker LA, Camphell PA. Thioglycollate medium decreases resistance to bacterial infection in mice. Infect Immunol 1980;27:455–60.
[6] Spitalny GL. Dissociation of bactericidal activity from other functions of activated macrophages in exudates induced by thioglycollate medium. Infect Immunol 1981;34:274–84.
[7] Wachsmuth ED. Aminopeptidase as a marker for macrophage differentiation. Exp Cell Res 1975;96:409–12.
[8] Wachsmuth ED, Stoye JP. Aminopeptidase on the surface of differentiating macrophages: induction and characterization of the enzyme. J Reticuloendothel Soc 1977;22:469–83.
[9] Murphy G, Gavrilovic J. Proteolysis and cell migration: creating a path. Curr Opin Cell Biol 1999;11:614–21.
[10] Foulon T, Cadel S, Cohen P. Aminopeptidase B (EC 3.4.11.6). Int J Biochem Cell Biol 1999;31(7):747–50.
[11] Silveira PF, Gil J, Casis L, Irazusta J. Peptide metabolism and the control of body fluid homeostasis. Curr Med Chem 2004;2:219–38.
[12] Jacotot E, Callebaut C, Blanco J, Krust B, Neubert K, Barth A, et al. Dipeptidyl-peptidase IV-beta, a novel form of cell-surface-expressed protein with dipeptidyl-peptidase IV activity. Eur J Biochem 1996;239:248–58.
[13] Kokkonen JO, Kuoppala A, Saarinen J, Lindstedt KA, Kovanen PT. Kallidin- and bradykinin-degrading pathways in human heart: degradation of kallidin by aminopeptidase M-like activity and bradykinin by neutral endopeptidase. Circulation 1999;99:1984–90.
[14] Palter SF, Mulayim N, Senturk L, Arici A. Interleukin-8 in the human fallopian tube. J Clin Endocrinol Metab 2001;86:2660–7.
[15] Tanioka T, Hattori A, Masuda S, Nomura Y, Nakayama H, Mizutani S. Human leukocyte-derived arginine aminopeptidase. The third member of the oxytocinase subfamily of aminopeptidases. J Biol Chem 2003;278:32275–83.
[16] Ziaber J, Bah Z, Pasnik J, Chmielewski H, Tchorzewski H. Expression of aminopeptidase N (APN) on peripheral blood mononuclear cells' surface as a marker of these cells' transendothelial migratio properties in the course of multiple sclerosis. Mediat Inflam 2000;9:45–8.

[17] Green GD, Shaw E. A prolyl endopeptidase from murine macrophages, its assay and specific inactivation. Arch Biochem Biopphys 1983;225:331–4.
[18] Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
[19] Bergmeyer HU, Brent E. Assay with pyruvate and NADH In Methods in enzymatic analysis, 2. London: Academic Press Inc., 1972 . p. 574–7.
[20] Ramírez-Expósito MJ, Martínez JM, Prieto I, Alba F, Ramírez M. Comparative distribution of glutamyl and aspartyl aminopeptidase activities in mouse organs. Horm Metab Res 2000;32:161–3.
[21] Silveira PF, Schiripa LN, Picarelli ZP. Hydrolysis of L-cystine-di-β-naphthylamide and neurohypophyseal peptides by the plasma of the snake Bothrops jararaca. Comp Biochem Physiol B 1992;102: 119–22.
[22] Mantle D. Comparison of soluble aminopeptidases in human cerebral cortex, skeletal muscle and kidney tissues. Clin Chim Acta 1992;207:107–18.
[23] Schwabe C, McDonald JK. Demonstration of a pyroglutamyl residue at the N terminus of the B-chain of porcine relaxin. Biochem Biophys Res Commun 1977;74:1501–4.
[24] Liu WJ, Hansen PJ. Progesterone-induced secretion of dipeptidyl peptidase-IV (cluster differentiation antigen-26) by the uterine endometrium of the ewe and cow that co-stimulates lymphocyte proliferation. Endocrinology 1995;136:779–87.
[25] Zolfaghari R, Baker CR, Canizaro PC, Feola M, Amirgholami A, Behal FJ. Human lung post-proline endopeptidase: purification and action on vasoactive peptides. Enzyme 1986;36:165–78.
[26] Erbeznik H, Hersh LB. A novel mammalian high-molecular-weight aminopeptidase. Arch Biochem Biophys 1997;344:228–34.
[27] Thompson MW, Tobler A, Fontana A, Hersh LB. Cloning and analysis of the gene for the human puromycin-sensitive aminopeptidase. Biochem Biophys Res Commun 1999;258:234–40.
[28] Cummins PM, O'Connor B. Pyroglutamyl peptidase: an overview of the three known enzymatic forms. Biochim Biophys Acta 1998;1429:1–17.
[29] Hui KS, Saito M, Hui M. A novel neuron-specific aminopeptidase in rat brain synaptosomes. Its identification, purification, and characterization. J Biol Chem 1998;273:31053–60.
[30] Murray H, Turner AJ, Kenny AJ. The aminopeptidase activity in the human T-cell lymphoma line (Jurkat) is not at the cell surface and is not aminopeptidase N (CD-13). Biochem J; 298:353–60.
[31] Yamamoto Y, Li YH, Ushiyama I, Nishimura A, Ohkubo I, Nishi K. Puromycin-sensitive alanyl aminopeptidase from human liver cytosol: purification and characterization. Forensic Sci Int 2000; 113:143–6.
[32] Kawai M, Otake Y, Hara Y. High-molecular-mass isoform of aminopeptidase N/CD13 in serum from cholestatic patients. Clin Chim Acta 2003;330:141–9.
[33] Bolumar T, Sanz Y, Aristoy MC, Toldra F. Purification and properties of an arginyl aminopeptidase from Debaryomyces hansenii. Int J Food Microbiol 2003;86:141–51.
[34] Ino K, Nagasaka T, Okamoto T, Uehara C, Nakazato H, Nakashima N. Expression of aminopeptidase A in human gestational choriocarcinoma cell lines and tissues. Placenta 2000;21:63–72.
[35] Ronai AZ, Timar J, Mako E, Erdo F, Gyarmati Z, Toth G, et al. Diprotin A, an inhibitor of dipeptidyl aminopeptidase IV(EC 3.4.14.5) produces naloxone-reversible analgesia in rats. Life Sci 1999;64: 145–52.
[36] Sato Y. Aminopeptidases and angiogenesis. Endothelium 2003;10: 287–90.
[37] VanHensbergen Y, Broxterman HJ, Peters E, Rana S, Elderkamp YW, van Hinsbergh VW. Aminopeptidase inhibitor bestatin stimulates microvascular endothelial cell invasion in a fibrin matrix. Thromb Haemost 2003;90:921–9.
[38] Yamamoto Y, Li YH, Ushiyama I, Nishimura A, Ohkubo I, Nishi K. Puromycin-sensitive alanyl aminopeptidase from human liver cytosol: purification and characterization. Forensic Sci Int 2000; 113:143–6.
[39] Breen G, Harwood AJ, Gregory K, Sinclair M, Collier D, St Clair D, et al. Two peptidase activities decrease in treated bipolar disorder not schizophrenic patients. Bipolar Disord 2004;6:156–61.
[40] Grdisa M, Vitale L. Types and localization of aminopeptidases in different human blood cells. Int J Biochem 1991;23:339–45.
[41] Heemels MT, Ploegh H. Generation, translocation, and presentation of MHC class I-restricted peptides. Annu Rev Biochem 1995;64: 463–91.
[42] Gibson AM, McDermott JR, Lauffart B, Mantle D. Specificity of action of human brain alanyl aminopeptidase on Leu-enkephalin and dynorphin-related peptides. Neuropeptides 1989;13:259–62.
[43] Perone MJ, Castro MG. Prohormone and proneuropeptide synthesis and secretion. Histol Histopathol 1997;12:1179–88.
[44] Chapman Jr HA.. Role of enzyme receptors and inhibitors in regulating proteolytic activities of macrophages. Ann NY Acad Sci 1991;624:87–96.
[45] Bauvois B, Djavaheri-Mergny M, Rouillard D, Dumont J, Wietzerbin J. Regulation of CD26/DPPIV gene expression by interferons and retinoic acid in tumor B cells. Oncogene 2000;19:265–72.
[46] Shane R, Wilk S, Bodnar RJ. Modulation of endomorphin-2 induced analgesia by dipeptidyl peptidase IV. Brain Res 1999;815:278–86.
[47] Ahmad S, Wang L, Ward PE. Dipeptidyl (amino) peptidase IV and aminopeptidase M metabolize circulating substance P in vivo. J Pharmacol Exp Ther 1992;260:1257–61.
[48] Ghersi G, Chen W, Lee EW. Critical role of dipeptidyl peptidase IV in neuropeptide Y-mediated endothelial cell migration in response to wounding. Peptides 2001;22:453–8.
[49] Abbott CA, McCaughan GW, Levy MT, Church WB, Gorrell MD. Binding to human dipeptidyl peptidase IV by adenosine deaminase antibodies that inhibit ligand binding involves overlap discontinuous sites on a predicted beta propeller domain. Eur J Biochem 1999;266:798–810.
[50] Mizutani S, Goto K, Nomura S, Ino K, Goto S, Kikkawa F, et al. Possible action of human placental aminopeptidase N in feto-placental unit. Res Commun Chem Pathol Pharmacol 1993;82:65–80.
[51] Larsen SL, Pedersen LO, Buus S, Stryhn A. T-cell responses affected by aminopeptidase N (CD13)-mediated trimming of major histocompatibility complex class II-bound peptides. J Exp Med 1996;184: 183–9.
[52] Weber M, Uguccioni M, Baggiolini M, Clark-Lewis I, Dahinden CA. Deletion of the NH_2-terminal residue converts monocyte chemotactic protein 1 from an activator of basophil mediator release to an eosinophil chemoattractant. J Exp Med 1996;183:681–5.
[53] Riemann D, Kehlen A, Thiele K, Lohn M, Langner J. Induction of aminopeptidase N/CD13 on human lymphocytes after adhesion to fibroblast-like synoviocytes, endothelial cells, epithelial cells, and monocytes/macrophages. J Immunol 1997;158:3425–32.
[54] Harris CA, Hunte B, Krauss MR, Taylor A, Epstein LB. Induction of leucine aminopeptidase by interferon-gamma. Identification by protein microsequencing after purification by preparative two-dimensional gel electrophoresis. J Biol Chem 1992;267:6865–9.
[55] VanHal PTW, Hopstaken-Broos JPM, Prins A, Favarolo EJ, Huijbens RJ, Hilvering C, et al. Potential indirect anti-inflammatory effects of IL-4. J Immunol 1993;153:2718–28.
[56] Hart SP, Ross JA, Ross K, Haslett C, Dransfield I. Molecular characterization of the surface of apoptotic neutrophils: implications for functional downregulation and recognition by phagocytes. Cell Death Differ 2000;7:493–503.
[57] Nissen JB, Iversen L, Kragballe K. Characterization of the aminopeptidase activity of epidermal leukotriene A4 hydrolase against the opioid dynorphin fragment 1-7. Br J Dermatol 1995;133:742–9.
[58] Wilk S. Prolyl endopeptidase. Life Sci 1983;33:2149–57.

[59] O'Leary RM, O'Connor B. Identification and localisation of a synaptosomal membrane prolyl endopeptidase from bovine brain. Eur J Biochem 1995;227:277–83.
[60] Wright JW, Mizutani S, Murray CE, Amir HZ, Harding JW. Aminopeptidase-induced elevations and reductions in blood pressure in the spontaneously hypertensive rat. Hypertens 1990;8:969–74.
[61] Fournie-Zaluski MC, Fassot C, Valentin B, Djordjijevic D, Reaux-Le Goazigo A, Corvol P, et al. Brain renin-angiotensin system blockade by systemically active aminopeptidase A inhibitors: a potential treatment of salt-dependent hypertension. Proc Natl Acad Sci USA 2004;101:7775–80.
[62] Sakurada C. Development of a new analgesic based on metabolism of endomorphin, an endogenous opioid peptide. Yakugaku Zasshi 2004;124:549–54.
[63] van Hensbergen Y, Broxterman HJ, Peters E, Rana S, Elderkamp YW, van Hinsbergh VW. Aminopeptidase inhibitor bestatin stimulates microvascular endothelial cell invasion in a fibrin matrix. Thromb Haemost 2003;90:921–9.
[64] Chakir M, D'Orleans-Juste P, Plante GE. Neutral endopeptidase inhibition, a new approach in the exploration of diabetic vasculopathy in rats. Eur J Pharmacol 1995;285:11–8.