Modulation of substance P signaling by dipeptidyl peptidase-IV enzymatic activity in human glioma cell lines

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Short title: Modulation of SP signaling in glioma cells by DPP-IV

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Summary

Dipeptidyl peptidase-IV (DPP-IV, CD26) is a serine protease almost ubiquitously expressed on cell surface and present in body fluids. DPP-IV has been suggested to proteolytically modify a number of biologically active peptides including substance P (SP) and the chemokine stromal cell derived factor-1α (SDF-1α, CXCL12). SP and SDF-1α have been implicated in the regulation of multiple biological processes and also induce responses that may be relevant for glioma progression. Both SP and SDF-1α signal through cell surface receptors and use intracellular calcium as a second messenger. The effect of DPP-IV on SP and SDF-1α mediated mobilization of intracellular calcium was monitored in suspension of wild type U373 and DPP-IV transfected U373DPPIV glioma cells using the ratiometric indicator FURA-2. Nanomolar concentrations of SP triggered a transient dose dependent increase in intracellular calcium rendering the cells refractory to repeated stimulation, while SDF-1α had no measurable effect. SP signaling in DPP-IV overexpressing U373DPPIV cells was not substantially different from that in wild type cells. However, preincubation of SP with the DPP-IV overexpressing cells lead to the loss of its signaling potential, which could be prevented with DPP-IV inhibitors. Taken together, DPP-IV may proteolytically inactivate local mediators involved in gliomagenesis.

Key words: dipeptidyl peptidase, substance P, glioma, NK1 receptor, calcium signaling

Introduction

Dipeptidyl peptidase-IV (DPP-IV, CD26) is a widely expressed 240 kDa serine protease with a multitude of functions under both physiological and pathological conditions (see Lambeir *et al.* 2003 for review). Its relatively restricted substrate specifity for Pro or Ala in the P1 position directs its action on a number of biologically active peptides such as neuropeptide Y, Substance P (SP), glucagon like peptide-1 and -2 and a number of chemokines including stromal cell derived factor-1α (SDF-1α, CXCL12) (Mentlein 1999, de Meester *et al.* 2000). Proteolytic removal of the two N-terminal amino acid residues by DPP-IV mostly decreases the biological activity of the respective peptide, while in some cases it can activate the peptide substrate or influence its binding to specific receptor subtypes (Mentlein 1999). DPP-IV is mostly expressed on cell surface and a soluble form is also present in the serum (Durinx *et al.* 2000). DPP-IV enzymatic activity can therefore affect auto-, para- as well as endocrine signaling of biologically active substances.

DPP-IV is frequently dysregulated in cancer, being significantly downregulated or lost in some tumors and upregulated in others (Sulda *et al.* 2006). A number of DPP-IV substrates promote the malignant phenotype of cancer cells that express appropriate receptors. Thus, DPP-IV hydrolytic activity can interfere with some pro-oncogenic signaling pathways (Busek *et al.* 2004, Busek *et al.* 2006). Indeed, Masur *et al.* (2006) have recently shown that a DPP-IV inhibitor can promote the growth stimulating and migratory effect of glucagon like peptide-2 in DPP-IV positive colon cancer cell lines. Interestingly, *in vitro* studies have mostly demonstrated that artificial upregulation of DPP-IV has an antioncogenic effect (Wesley *et al.* 1999, 2004, 2005), although the mechanism remains elusive.

In brain tumors, DPP-IV substrates SP and SDF-1α trigger a number of intracellular signaling cascades that affect cell proliferation, survival, migration and invasion (Barbero *et al.* 2003,

Palma and Maggi 2000, Sharif 1998). Indeed, antagonists of the respective receptors NK1 and CXCR4 exhibit significant antitumor activity in gliomas (Palma *et al.* 2000, Rubin *et al.* 2003).

We have previously detected expression and activity of DPP-IV and possibly other molecules bearing similar enzymatic activity (Fibroblast activation protein-α, dipeptidyl peptidases 8 and 9) in human glioma tumors and cell lines (Sedo *et al.* 2004, Stremenova *et al.* 2006). Here we explore, whether DPP-IV enzymatic activity can influence signaling of selected biologically active DPP-IV substrates in glioma cell lines.

Material and Methods

Chemicals and cell lines

Human SDF-1α was purchased from PeproTech (UK), SP, Diprotin A and Lys[Z(NO2)]-pyrrolidide were from Bachem (Switzerland). Glioma cell lines U373 and T98G (ATCC, USA) and their transfectants (U373DPPIV, T98GDPPIV) were cultured in Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum (Sigma, Czech Republic) under standard conditions. DPP-IV was transfected into U373 and T98G cells using the mifepristone inducible Gene Switch system (Invitrogen, USA). The transfectants inducibly expressed DPP-IV upon treatment with 5 nM Mifepristone (mife). Maximum DPP-IV upregulation was achieved after 24 hours in U373DPPIV and after 48 hours in T98GDPPIV cells.

Real time RT-PCR

Total RNA was isolated using the TriZol Reagent (Invitrogen, UK) according to the manufacturer's instructions. Spectrophotometric analysis, carried out in 10 mmol/l Tris/HCl buffer, pH 7.5, revealed that the samples of total RNA had an A260 nm/A280 nm ratio greater

than 1.8. The concentration of total RNA was determined using the RiboGreen RNA Quantitation Kit (Molecular Probes, Eugene, USA).

Gene coding region-specific oligonucleotide primers and fluorogenic TaqMan probes for the real time RT-PCR assays of expression of the investigated transcripts were designed with the program Primer Express (Applied Biosystems, USA) and were synthesized at Proligo (France) and Applied Biosystems (UK), respectively (Table 1). The expression of DPP-IV, NK1 receptor, CXCR4 and β-actin mRNA (an internal reference transcript) was quantified by coupled real time RT-PCR assays. The RT-PCR reaction mixtures of a total volume of 50 µl contained 25 µl of ThermoScript Reaction Mix (a buffer with 3 mmol/l $MgSO_4$ and 0.2 mmol/l of each dGTP, dCTP, dATP and dTTP) and 1 µl of ThermoScript Plus Reverse Transcriptase/Platinum Taq DNA polymerase Mix (both Mixes were from Platinum Quantitative RT-PCR ThermoScript One-Step System, Invitrogen), the respective gene-specific primers and TaqMan probe, 40 units of RNase inhibitor RNaseOUT (Invitrogen), and an input of 200 ng of total RNA. The real time RT-PCR assays were run in duplicate in MicroAmp Optical 96-well Reaction Plates on the ABI PRISM 7700 Sequence Detection System operated by the Sequence Detection System software (Applied Biosystems). The reverse transcription was carried out at 58 °C for 30 min and the subsequent PCR amplification included a hot start at 95 °C for 5 min and 45 cycles of denaturation at 95 °C for 15 s and of annealing/extension at 58 °C for 1 min. The threshold cycle (Ct) values of the amplification reactions, represented by the plots of background-subtracted fluorescence intensity (ΔFI) of the reporter dye (6-FAM or VIC) against PCR cycle number, were determined with the Sequence Detection System software. Target transcript expression was normalized to β-actin mRNA expression using the ΔCt method and the linearized ΔCt (i.e. $2^{-\Delta Ct}$) was used for comparative purposes (Livak and Schmittgen 2001).

DPP-IV enzymatic activity assay

Cell surface DPP-IV enzymatic activity was assessed in suspensions of viable cells by a continuous rate fluorimetric assay using 7-(glycyl-L-prolylamido)-4-methylcoumarin (Gly-Pro-AMC; Bachem, Switzerland) as a substrate at a pH of 7.5 and 37 °C (Sedo *et al.* 1989).

Measurement of intracellular calcium

Mobilization of intracellular Ca^{2+} was monitored in cell suspensions at 37 °C after loading cells with the ratiometric indicator FURA-2 (final concentration [f.c.] 4 μmol/l) for 30 min and scraping the cells gently into Krebbs buffer (120 mmol/l NaCl, 4.75 mmol/l KCl, 1 mmol/l KH_2PO_4, 5 mmol/l $NaHCO_3$, 1.44 mmol/l $MgSO_4$, 1.1 mmol/l $CaCl_2$, 0.1 mmol/l EGTA, 11 mmol/l glucose, 25 mmol/l NaHEPES, 0.1% bovine serum albumin fraction V, pH 7.4). Fluorescence was measured at 340 nm/380 nm (excitation) and 510 nm (emission) on a Perkin Elmer spectrofluorometer. Stock solution of SP was added to the cuvette with tested cells either directly or after 10-30 minutes of preincubation at 37 °C with T98GDPPIV cells induced or not induced to upregulate DPP-IV. Concentration of intracellular calcium was calculated using the equation $[Ca^{2+}]_i = K_d \times (R-R_{min})/(R_{max}-R) \times SFB$, where R is the emission ratio value (340 nm/380 nm). R_{max}, the maximum 340 nm/380 nm ratio, was determined by lysing the cells with 0.1% Triton X-100 in the presence of 1 mmol/l $CaCl_2$. The R_{min} 340 nm/380 nm ratio was obtained by adding 40 mmol/l EGTA. K_d is the dissociation constant of the Fura-2/Ca^{2+} complex (225 nmol/l) and SFB is the ratio of 380 nm fluorescences under Ca^{2+}-free/Ca^{2+} saturated conditions. Trypsin, which is known to induce calcium oscillations in glioma cells (Ubl *et al.* 1998), was used at a f.c. of 10^{-4} g/l as a positive control for the above described $[Ca^{2+}]_i$ rise measuring method.

Results

To quantify the potential of our model cell lines to proteolytically process biologically active DPP-IV substrates, cell surface DPP-IV enzymatic activity was determined in wild type U373 and T98G cells and in their transfected counterparts U373DPPIV and T98GDPPIV. Cell surface DPP-IV enzymatic activity was upregulated upon mifepristone stimulation ten times in U373DPPIV and several hundred times in T98GDPPIV in comparison with the respective mifepristone unstimulated transfectants and wild type cells. Upregulation of DPP-IV was also confirmed on transcriptional level (Table 2). We also determined the expression of transcripts of NK1 receptor and CXCR4, the receptors of SP and SDF-1α, respectively (Table 2).

We then tested the ability of DPP-IV substrates SP and SDF-1α to trigger calcium signaling in our model cell lines. SP caused a concentration dependent transient rise of $[Ca^{2+}]_i$ in U373 rendering the cells refractory to repeated stimulation (Fig.1). This rise of $[Ca^{2+}]_i$ induced by SP was however not significantly affected by upregulation of DPP-IV in U373DPPIV cells (data not shown). To assess the possible effect of prolonged exposure of SP to high DPP-IV activity, U373 cells were exposed to SP preincubated with DPP-IV upregulating T98GDPPIV glioma cells. Such pre-treatment of SP abrogated its signaling potential (Fig 2b). SP exposed to DPP-IV upregulating T98GDPPIV cells also lost its capacity to induce the abovementioned refracterity of U373 to repeated stimulation by SP not exposed to DPP-IV enzymatic cleavage (Fig. 2b, c, f). On the contrary, preincubation of SP with T98GDPPIV cells not upregulating DPP-IV did not affect its potential to trigger calcium signaling (Fig. 2a). DPP-IV inhibitors Diprotin A and Lys[Z(NO2)]-pyrrolidide preserved the signaling potential of SP, which confirms that SP was inactivated specifically by DPP-IV enzymatic activity (Fig. 2d-f).

SDF-1α did not cause measurable changes of $[Ca^{2+}]_i$ in any cell line tested (data not shown).

Discussion

DPP-IV is a widely expressed serine protease that can proteolytically modify a number of biologically active peptides. It is considered to be an important regulator of SP plasma half-life (Ahmad *et al.* 1992, Wang *et al.* 1991) and has been shown to affect some SP regulated physiological processes (Grouzmann *et al.* 2002, Guieu *et al.* 2006). Functional SP receptors NK1 are widely expressed in astrocytic brain tumors and have been implicated in the regulation of their growth (Palma *et al.* 2000, Sharif 1998). NK1 receptor triggers a number of signaling cascades including elevation of $[Ca^{2+}]_i$ and activation of mitogen-activated protein kinases that can mediate the growth promoting effect of SP (Luo *et al.* 1996, Palma *et al.* 1999).

We previously detected DPP-IV enzymatic activity in human astrocytic tumor tissues and glioma cell lines (Sedo *et al.* 2004, Stremenova *et al.* 2006) and observed decreased growth of DPP-IV upregulating transfectants (Busek *et al.* 2006). Here we demonstrate that DPP-IV enzymatic activity can influence the signaling potential of SP in glioma cell lines. In agreement with the literature (Sharif 1998, Palma *et al.* 1999), SP induced Ca^{2+} signaling in U373 but not in T98G cell line, which well corresponds to the observed substantially lower expression of NK1 receptor mRNA in T98G cells (Table 2). SDF-1α on the contrary did not cause measurable changes of $[Ca^{2+}]_i$ in any cell line tested, although we detected mRNA expression of its receptor CXCR4. However, also Oh *et al.* (2001) could observe SDF-1α induced changes of $[Ca^{2+}]_i$ in glioma cells only after enhancement of CXCR4 expression by cytokines.

Calcium signaling triggered by SP in U373DPPIV cells upregulating DPP-IV was similar in duration and magnitude to that in wild type U373. This is most likely because the second messenger calcium signaling occurs within seconds while degradation of SP by cell surface DPP-IV into the less active SP5-11 fragment may require longer periods of time. Also, the

upregulation of DPP-IV was much lower in U373DPPIV compared to T98GDPPIV, which on the other hand did not express NK1 receptors (Table 2.). However, it should be considered that (i) complex cellular programs such as cell proliferation frequently require prolonged exposure to the particular ligand, and (ii) the ligand may be subject to functionally relevant proteolytic cleavage by surrounding cells that neither secrete nor respond to it. To simulate functional interaction of two cell types in SP signaling**,** U373 cells were exposed to SP that had been preincubated with T98GDPPIV glioma cells overexpressing DPP-IV but lacking NK1 receptor. These experiments demonstrated functional inactivation of SP, which was prevented with specific DPP-IV inhibitors.

Cleavage of SP and other susceptible regulatory molecules may contribute to the growth inhibitory effect of DPP-IV that has been observed in several cell lines (Wesley *et al.* 1999, 2004, 2005), including glioma cells (Busek *et al.* 2006). Our results also suggest that prereceptor modification of signaling peptides by DPP-IV enzymatic activity may be physiologically relevant even for DPP-IV negative cells. Thus, DPP-IV present in the tissue microenvironment may represent an important regulator of local humoral signaling.

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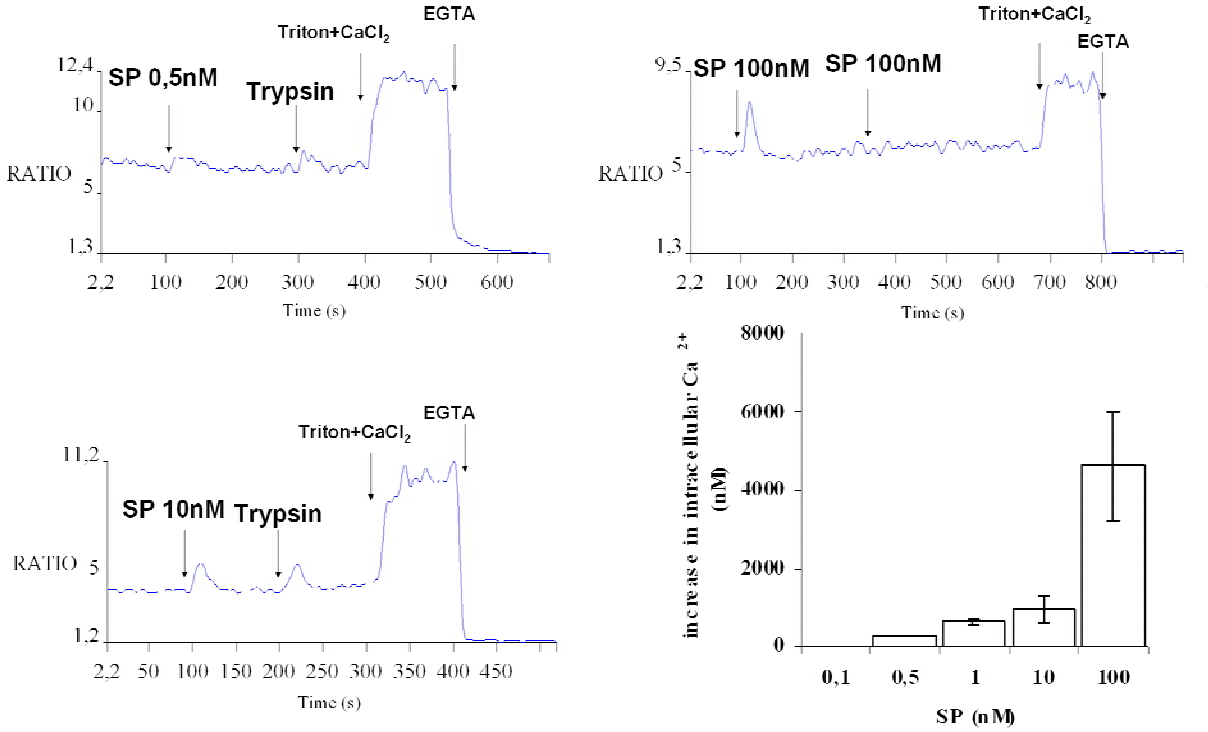

Figure 1. Substance P triggers concentration dependent mobilization of intracellular calcium in U373 cell line

Intracellular Ca^{2+} release was measured using the fluorescent probe Fura-2 AM. The fluorescence ratio at the two excitation wavelengths (vertical axis) is presented as an indicator of intracellular Ca^{2+} mobilization. Intracellular Ca^{2+} concentration was calculated as described in Materials and Methods; bars represent means ± SEM. SP: substance P, concentration in nmol/l [nM]

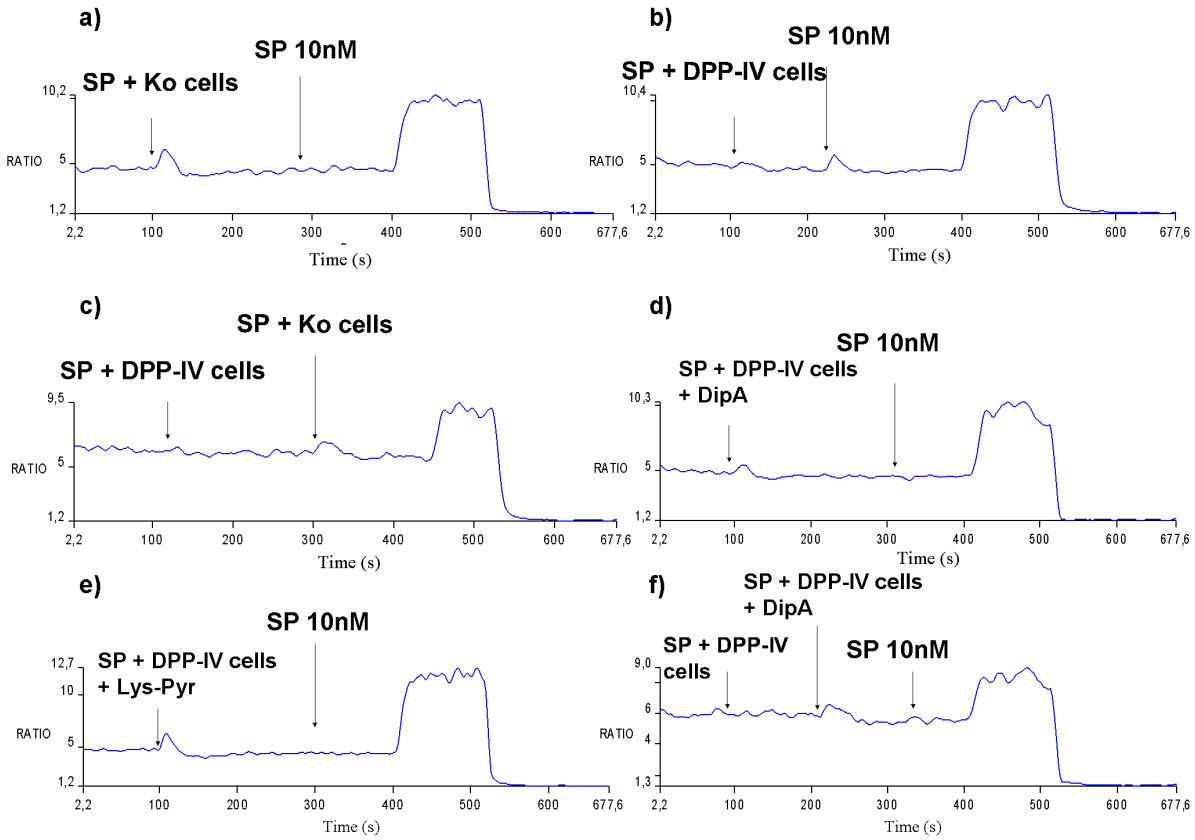

Figure 2. Substance P loses its ability to trigger calcium signaling in U373 cells after preincubation with DPP-IV overexpressing cells

Intracellular Ca^{2+} release was monitored as described in Fig.1. Substance P (SP, 10 nmol/l [nM]) was preincubated in the suspension of T98GDPPIV cells that (a) had not been (Ko cells) or (b) had been (DPP-IV cells) induced to overexpress DPP-IV. Plots (d), (e), (f): indicated DPP-IV inhibitors DipA (200 µmol/l) and Lys-Pyr (200 µmol/l) were present in the preincubation mixture. Neither T98GDPPIV cells nor inhibitors alone induced calcium signaling in U373 cell line (data not shown). DipA: Diprotin A; Lys-Pyr: Lys[Z(NO2)]-pyrrolidide.

Table 1. Primers and TaqMan probes used for real time RT-PCR quantitation of expression of the investigated transcripts

Transcript	GeneBank Accession No.	Sequences and final concentration of primers and TaqMan probes
DPP-IV	NM_001935	Forward primer: 5'-TGGAAGGTTCTTCTGGGACTG-3', 200 nmol/l Reverse primer: 5'-GATAGAATGTCCAAACTCATCAAATGT-3', 200 nmol/l TaqMan probe: 5'-(6-FAM)CACCGTGCCCGTGGTTCTGCT(TAMRA)-3', 200 nmol/l
NK1	NM_001058	Forward primer: 5'-CAGTGGTGAACTTCACCTATGCT-3', 400 nmol/l Reverse primer: 5'-GATGTATGATGGCCATGTACCTATC-3', 400 nmol/l TaqMan probe: 5'-(6-FAM)TCCACAACTTCTTTCCCATCGCCG(TAMRA)-3', 200 nmol/l
CXCR4	NM_001008540	Forward primer: 5'-CATGGGTTACCAGAAGAAACTGA-3', 400 nmol/l Reverse primer: 5'-GACTGCCTTGCATAGGAAGTTC-3', 400 nmol/l TaqMan probe: 5'-(6-FAM)CACCTGTCAGTGGCCGACCTCCT(TAMRA)-3', 200 nmol/l
β-Actin	NM_001101	Forward primer: 5'-CTGGCACCCAGCACAATG-3', 200 nmol/l Reverse primer: 5'-GGGCCGGACTCGTCATAC-3', 200 nmol/l TaqMan probe: 5'-(VIC)AGCCGCCGATCCACACGGAGT(TAMRA)-3', 200 nmol/l

Table 2. Expression of DPP-IV and receptors of its biologically active substrates in wild type glioma cell lines (U373 and T98G) and transfected cells (U373DPPIV and T98GDPPIV) stimulated (mife +) or not stimulated (mife -) to express DPP-IV.

	Relative cell surface DPP-IV enzymatic activity/10^6 cells	**Relative mRNA expression ($2^{-\Delta Ct}$) x10^{-3}**		
		DPP-IV	**NK1**	**CXCR4**
Wild cells				
U373	1,0 ± 0,1	3,148 ± 1,138	1,129 ± 0,338	0,627 ± 0,055
T98G	0,7 ± 0,1	0,004 ± 0,001	0,011 ± 0,003	0,286± 0,067
Transfected cells				
U373DPPIVmife -	2,4 ± 0,1	89,630 ± 17,872	2,314 ± 0,628	0,195 ± 0,067
U373DPPIV mife +	27,4 ± 0,5	663,675 ± 63,162	5,097 ± 1,062	0,665 ± 0,095
T98GDPPIV mife -	1,3 ± 0,1	36,818 ± 10,271	0,143 ± 0,083	0,078 ± 0,042
T98GDPPIV mife +	543,7 ± 90,0	2444,720 ± 326,319	0,005 ± 0,001	0,074 ± 0,026

The expression of DPP-IV, NK1 receptor and CXCR4 mRNAs was normalized to the expression of human β-actin mRNA. Data are presented as means ± SEM of at least three measurements.