

# Nerve growth factor and receptors are significantly affected by histamine stimulus through H<sub>1</sub> receptor in pancreatic carcinoma cells

ZHI-YU WANG<sup>1,6</sup>, YAN DING<sup>2</sup>, TAKANORI MIKI<sup>6</sup>, KATSUHIKO WARITA<sup>6</sup>, YOSHIKI MATSUMOTO<sup>6</sup>,  
YOSHIKI TAKEUCHI<sup>6</sup>, SHI-JIE WANG<sup>1</sup>, JIAN-GANG FENG<sup>3</sup>, WEI LIU<sup>4</sup>, YA-DI WANG<sup>5</sup>,  
XIAO-LING WANG<sup>2</sup>, YU-HUA WANG<sup>4</sup>, YA LIU<sup>1</sup> and BAO-EN SHAN<sup>1</sup>

<sup>1</sup>Scientific Research Centre, Hebei Medical University Fourth Hospital, Departments of <sup>2</sup>Pathology, <sup>3</sup>Surgery,

<sup>4</sup>Medical Oncology, and <sup>5</sup>Radiotherapy, Hebei Medical University Fourth Hospital, Shijiazhuang, Hebei 050011, P.R. China;

<sup>6</sup>Department of Anatomy and Neurobiology, Faculty of Medicine, Kagawa University, Kagawa 761-0793, Japan

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**Abstract.** Nerve growth factor (NGF) as an autocrine or paracrine growth factor plays a critical role in the pathogenesis and progression of human pancreatic cancer. NGF is synthesized as a proform (proNGF) that, when cleaved, releases mature ligand (mNGF). proNGF and mNGF bind to high-affinity tyrosine kinase receptor A (TrkA) and low-affinity receptor p75 to different extents. Histamine is a potent stimulator of NGF in the inflammatory lesion as determined by ELISA. This has generally been attributed to the accumulation of mNGF. To determine the effect of histamine on nerve growth factor/receptor expression in human pancreatic cancer, the present study explored intracellular and extracellular NGF production and p75 and TrkA membrane receptor expression in the PANC-1, KMP-6 and PK-1 cell lines. Histamine enhanced NGF secretion and mRNA expression in PANC-1 and KMP-6 cells, but not in PK-1 cells. proNGF was revealed using Western blotting to be the predominant form of NGF, but was significantly reduced by histamine. p75 receptor binding was increased with histamine treatment, but no significant alteration was observed for TrkA. Proliferating cell nuclear antigen (PCNA), an important indicator of cell proliferation, was significantly reduced by histamine stimulation. H<sub>1</sub> and H<sub>2</sub> receptors were both observed in the pancreatic cancer cells, and the alterations induced by histamine were counteracted by H<sub>1</sub> receptor antagonist pyrilamine; however, the H<sub>2</sub> receptor subtype was excluded from this process. These results suggest that histamine induces distinct nerve

growth factor/receptor responsiveness via H<sub>1</sub> receptor-induced signaling, thus affecting pancreatic cancer cell proliferation.

## Introduction

Nerve growth factor (NGF) is a critical member of the neurotrophin family involved in pancreatic ductal adenocarcinoma (PDAC) cell survival, differentiation and death (1). NGF is translated from two major alternatively spliced transcripts to produce pre-proproteins, a long transcript termed pre-proNGF 34 kDa and a short transcript termed pre-proNGF 27 kDa. Removal of the signal sequence reduces the translation products to proNGF species 32 kDa and 25 kDa, which were found to be the predominant form of NGF in the human brain (2). Precursor (proNGF) is proteolytically cleaved to generate mature biologically active NGF 13.2 kDa (2-4). The biological action of NGF is regulated by proteolytic cleavage, with its preform preferentially activating p75 to mediate apoptosis and its mature forms activating Trk receptors to promote survival (5,6). Recent *in vivo* research has demonstrated that proNGF is potentially responsible for much of the biological activity normally attributed to mature NGF (mNGF), despite its being less active than mNGF (7-9). p75 and TrkA are well known as important regulators of tumor growth, and are crucial to the progression of certain types of carcinoma cells (10-14). An imbalance in p75 and tyrosine kinase receptor A (TrkA)-mediated signaling may be involved in the progression of cancer cells through increased proliferation and reduced apoptosis (15-16). It has been demonstrated that, depending on the operative ligands, coexpression of TrkA and p75 is capable of promoting cell survival or death (17-20). proNGF may therefore act as a reservoir for mNGF when survival and/or differentiation signaling is needed, or as a facilitator of death pathways when apoptosis is required.

Histamine as a classic inflammatory mediator plays an important role in a number of processes, including inflammation, allergic reaction, neurotransmission and even tumor development and progression (21,22). The different biological

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**Correspondence to:** Professor Bao-En Shan, Scientific Research Centre, Hebei Medical University Fourth Hospital, No. 12 JianKang Road, Shijiazhuang City, HeBei 050011, P.R. China  
E-mail: hebeisbe@yahoo.com.cn

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Table I. Oligonucleotide sequence for PCR.

Gene	Sense/antisense	Length of PCR product (bp)	Gene accession no.	Position
NGF	5'-ATACAGGCGGAACCACACTC-3' 5'-TGCTCCTGTGAGTCCTGTTG-3'	312	NM_013609	3': 215-234 5': 527-508
p75	5'-TGAGTGCTGCAAAGCCTGCAA-3' 5'-TCTCATCCTGGTAGTAGCCGT-3'	229	NM_002507.1	3': 236-256 5': 465-445
TrkA	5'-CCATTTCACCTCCTCGGCTCAGT-3' 5'-GCAGACCCCCAGATTTCATCAC-3'	400	NM_002529.3	3': 389-410 5': 789-768
PCNA	5'-CAGGGGCTCCATCCTCAAGAA-3' 5'-TCTTCATTGCCGGCGCATT-3'	236	NM_002529.2	3': 261-280 5': 497-478
β-actin	5'-TCACCCACACTGTGCCCATCTACGA-3' 5'-CAGCGGAACCGCTCATTGCCAATGG-3'	291	NM_001101.2	3': 549-573 5': 840-819

RNA was reverse transcribed with Ready-To-Go You-Prime First-Strand Beads and Oligo dT primer; PCR was performed using the LightCycler system with SYBR Green I Master Mix Plus. PCR products produced a single band.

effects are mediated through its specific receptors, which are classified as H<sub>1</sub>, H<sub>2</sub>, H<sub>3</sub> and H<sub>4</sub> isotypes. It has been demonstrated that histamine is produced by PDAC cells and exerts its action as an autocrine growth factor to regulate cell proliferation through H<sub>1</sub> and H<sub>2</sub> membrane receptors (23). Previous studies using two-site ELISA reported an increase in NGF-like immunoreactivity in histamine-stimulated NGF synthesis and secretion from cultured astrocytes and human keratinocytes. This has on the whole been attributed to the accumulation of mNGF (24-26). However, the present study suggests the alternate possibility that proNGF is the principal form of NGF, and that histamine potentiates the release and maturation of proNGF in human pancreatic cancer cells. Additionally, the results indicate that the disruption of the balance between low-affinity receptor p75 and high-affinity receptor TrkA may be attributed to cancer cell proliferation.

## Materials and methods

**Cell culture and experimental reagents.** The human pancreatic cancer cell lines PANC-1, KMP-6 and PK-1 were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 µg/ml penicillin and 100 µg/ml streptomycin in a 5% CO<sub>2</sub> humidified atmosphere at 37°C. Primary antibodies for TrkA, p75 and proliferating cell nuclear antigen (PCNA) were obtained from Upstate Biotechnology (Waltham, MA, USA). Anti-NGF for mNGF and proNGF were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Donkey anti-rabbit secondary antibody conjugated with horseradish peroxidase (HRP) and a chemiluminescence detection kit were obtained from GE Healthcare (Buckinghamshire, UK). An ELISA assay for NGF secretion was purchased from Promega (Madison, WI, USA). Histamine dihydrochloride, H<sub>1</sub> antagonist pyrilamine, the H<sub>2</sub> antagonist cimetidine, protease inhibitor cocktail and the remaining chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

**Nerve growth factor secretion.** Cancer cells were grown in complete medium to 50-70% confluence, washed twice with PBS (pH 7.4), and then incubated in serum-free conditioned medium at 37°C in a 5% CO<sub>2</sub> environment for 24 h. The medium was removed and the cells were incubated with histamine or pre-treated with H<sub>1</sub> and H<sub>2</sub> antagonists in serum-free medium for 24 h. The culture medium was collected and ELISA was performed according to the manufacturer's instructions.

**Real-time RT-PCR.** Following incubation as described above, cellular RNA was extracted with available TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The concentration and purity of the extracted RNA were evaluated by optical density measurements at 260 nm and 280 nm using a spectrophotometer (NanoDrop ND-1000, Thermo Fisher Scientific, Inc., Rockford, IL, USA). Ready-To-Go You-Prime First-Strand Beads (GE Healthcare, Chalfont St. Giles, UK) were used for reverse transcription according to the manufacturer's instruction. Real-time PCR was performed using the LightCycler rapid thermal cycler system (Roche Diagnostics Ltd., Lewes, UK) with SYBR Green I Master Mix (Roche Diagnostics Ltd.). The amplification protocol consisted of one cycle at 95°C for 10 min followed by 45 cycles at 95°C for 10 sec, 59°C (NGF), 62°C (TrkA), 58°C (p75) or 57°C (PCNA) for 10 sec, and 72°C for 20 sec. Detection of the fluorescent products was carried out following an extension period at 55°C. β-actin was used as the appropriate internal control for each sample. The forward and reverse primers used are shown in Table I. To confirm amplification specificity, the PCR products from each primer pair were subjected to melting curve analysis and subsequent sequence analysis. For melting curve analysis, the PCR products were melted by gradually increasing the temperature from 65°C in 0.2°C steps. Electrophoresis of the PCR products amplified from cDNA using each primer was carried out on 2% agarose gel stained with ethidium bromide to exclude genomic contamination.

**Western blot analysis.** Pancreatic cancer cells were lysed in lysis buffer containing 50 mM Tris-base (pH 7.4), 150 mM NaCl, 0.5 mM sodium vanadate, 1% NP-40, 0.1 mM phenyl-methylsulphonyl fluoride, 1  $\mu$ g/ml aprotinin, 1  $\mu$ g/ml leupeptin supplemented with protease inhibitors and complete mini protease inhibitor cocktail (Roche, Indianapolis, IN, USA). The extracts were then centrifuged at 13,000 g for 20 min at 4°C. The total amount of protein in the supernatant was determined by the bicinchoninic (BCA) acid method using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. Subsequently, the supernatant was diluted 1:1 with sample buffer (Laemmli 2X Concentrate, Sigma-Aldrich) and stored at -20°C. Equal amounts of protein (80  $\mu$ g/lane) per sample were heated for 5 min at 100°C, cooled for 5 min on ice, and then resolved on 6-15% SDS-polyacrylamide gels. Following electrophoresis, proteins were transferred to an Immobilon-P transfer membrane (Millipore, Bedford, MA, USA). Membranes were probed overnight at 4°C with the following antibodies: anti-NGF for mNGF and proNGF (anti-rabbit polyclonal; 1:500; Santa Cruz Biotechnology, Inc.); anti-TrkA (anti-rabbit polyclonal; 1:1000; Upstate Biotechnology); anti-p75 (anti-rabbit polyclonal; 1:1000; Upstate Biotechnology); and anti-PCNA (anti-rabbit polyclonal; 1:3000; Calbiochem, Cambridge, MA, USA).  $\beta$ -actin was used as a control to determine whether the ethanol effects observed were selective or global. After rinsing with 0.05% TBS-T solution, the membranes were incubated with the secondary donkey anti-rabbit antibody conjugated with horseradish peroxidase (HRP) at a dilutions of 1:5,000 for 60 min at room temperature. Signals were detected by enhanced chemiluminescence (ECL, Amersham, UK), and signal strength was quantified by the LAS1000plus lumino-imaging analyzer (Fujix, Tokyo, Japan). Densitometric analysis was performed using the LAS-1000 luminescence analyzer (Fujifilm, Tokyo, Japan). Average background density was subtracted and optical density values were measured. Each sample was analyzed in duplicate to ensure consistent results.

**Statistical analysis.** The mean  $\pm$  standard error of the mean (SEM) was calculated for each group of cells. Statistical analysis comparing the experimental and control groups was performed by one-way ANOVA with the Dunnett's post hoc test. mRNA quantification data were analyzed with LightCycler analysis software. Data regarding NGF, TrkA, p75 and PCNA mRNA and protein were normalized with reference to the  $\beta$ -actin housekeeping gene. Statistical analysis was performed using SigmaStat software (Systat Software, version 3.0).

## Results

**Histamine dose-dependently increased NGF secretion through  $H_1$  receptor in pancreatic carcinoma cells.** As the expression of specific histamine membrane receptors (including  $H_1$ ,  $H_2$ ,  $H_3$ ,  $H_4$ ) differs among different tissues and cells, RT-PCR was used to determine that  $H_1$  and  $H_2$  were the main receptors located in the pancreatic cancer cells (Fig. 2C). Thus,  $H_1$  receptor antagonist pyrilamine and  $H_2$  receptor antagonist cimetidine were used. Pyrilamine and cimetidine did not alter constitutive NGF secretion in the absence of exogenous histamine (data not shown). In PK-1 cells, little NGF secretion could be found

whether the cells were treated with histamine or not (Fig. 1A). ELISA indicated that a low amount of NGF (mean  $\pm$  SEM for PANC-1, 83.48 $\pm$ 7.64 pg/ml; KMP-6, 40.25 $\pm$ 4.21 pg/ml; n=6) was constitutively secreted, and was concentration-dependently increased by histamine (Fig. 1C and D).  $H_1$  receptor antagonist pyrilamine blocked the stimulatory effect of histamine, whereas the  $H_2$  antagonist cimetidine did not. As shown in Fig. 1, a concentration of  $10^{-6}$  M histamine was optimal for NGF induction, and was used in the experiments. The time course of NGF secretion by histamine stimulation indicated that the maximal concentration of NGF in medium was observed at 24 h (Fig. 1B). ELISA indicated that constitutive histamine secretion by pancreatic cancer cells ( $\sim 10^{-10}$  M) was less than the threshold ( $10^{-9}$  M) for NGF induction. This suggests that endogenous histamine secreted by pancreatic cancer cells may not contribute to NGF secretion.

**Histamine altered nerve growth factor/receptor mRNA levels.** Fig. 2 shows the mean  $\pm$  SEM ratio of NGF, TrkA, p75 and PCNA mRNA to  $\beta$ -actin with and without histamine induction in PANC-1 and KMP-6 cells. PK-1 cells showed no significant alteration with histamine (data not shown). Quantitative real-time RT-PCR analysis demonstrated that incubation with histamine for 16 h resulted in significant alterations in the level of nerve growth factor ligands/receptors. As shown in Fig. 2, the NGF mRNA level was increased in histamine-treated compared to histamine-free cells. In conjunction with a change in NGF mRNA expression, a significant increase in low-affinity receptor p75 mRNA was observed in the cells exposed to histamine. For high-affinity receptor TrkA mRNA, no significant change was observed. By contrast, PCNA was suppressed by histamine induction. Alterations were blocked by pyrilamine but not by cimetidine (Fig. 1A and B). Real-time RT-PCR was performed as previously described (27-29). To verify whether specific amplification had been accomplished, melting curve analysis was used to verify that the PCR products amplified from the target and housekeeping genes had a single and sharp transition (data not shown), confirming that a single PCR product was present. Primer-dimer formation was a rare occurrence within the number of cycles required for quantification. Additionally, agarose gel electrophoresis of the PCR products showed a single band. When omitting reverse transcription, no amplification products were observed (data not shown).

**Effect of histamine on nerve growth factor/receptor protein levels in pancreatic cancer cells.** Fig. 3 shows the mean  $\pm$  SEM optical densities of NGF, p75, TrkA and PCNA proteins and representative Western blots of the histamine-treated and histamine-free groups. Western blot analysis revealed that incubation with histamine for 24 h resulted in significant alterations in the protein level of nerve growth factor ligands/receptors. Alterations in these proteins were profoundly correlated with alterations in mRNA levels observed in PANC-1 and KMP-6, but not in PK-1 cells (Fig. 3A and B). NGF protein in pancreatic cancer cells was present as a mixture of proNGF and mNGF, with proNGF predominating. As a result of incubation with histamine for 24 h, proNGF was significantly reduced (28.5% in PANC-1, 28% in KMP-6,  $p < 0.01$ ), while mNGF was distinctly enhanced (20% in PANC-1,

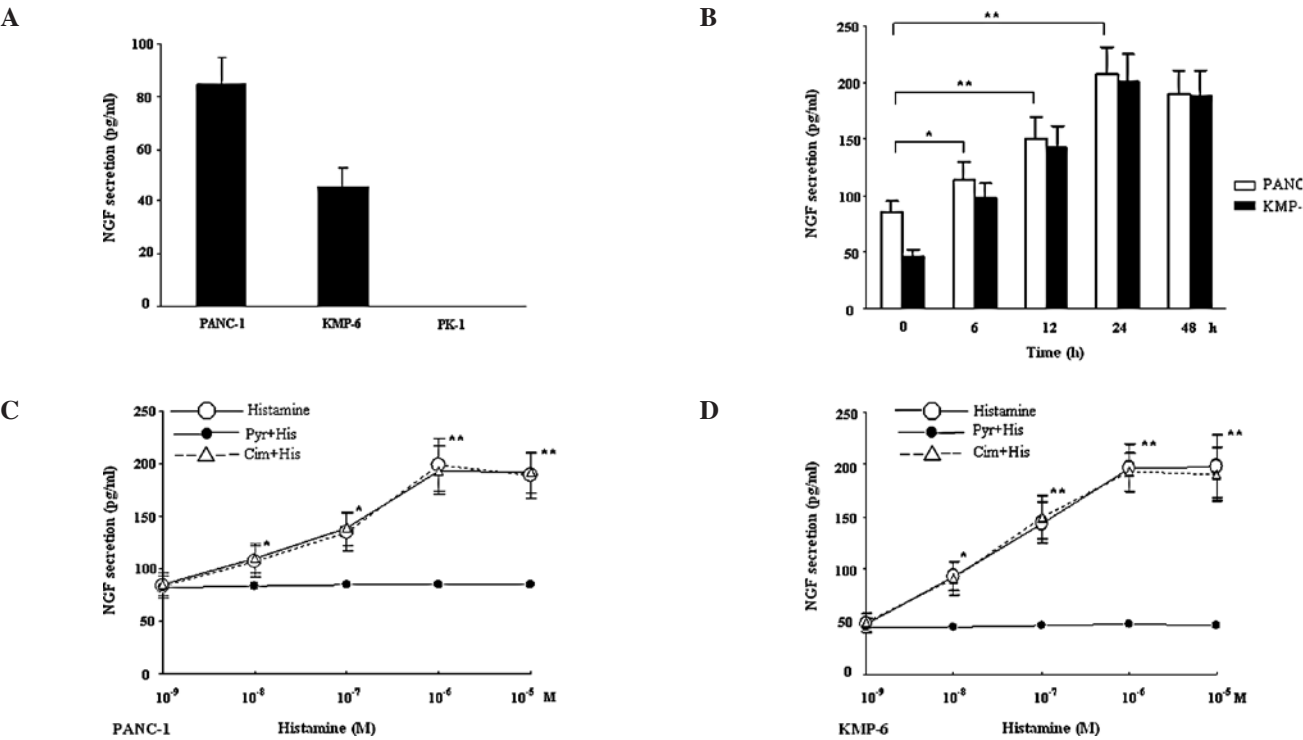


Figure 1. Dose-dependent increase in NGF secretion in cultured PDAC cells by histamine and its inhibition by H<sub>1</sub> receptor antagonists. (A) NGF basic secretion by three histamine-free pancreatic cancer cells. The cells were grown in complete medium to 50-70% confluence and then incubated in serum-free medium for 24 h. The supernatants were assayed for NGF by ELISA. (B) Time course of NGF production by histamine stimulation ( $10^{-6}$  M). The maximal concentration of NGF in medium was observed at 24-48 h in PANC-1 and KMP-6 cells after histamine stimulation (n=6, \*p<0.05, \*\*p<0.01). (C and D) PANC-1 and KMP-6 cells were pre-incubated with pyrilamine (Pyr) or cimetidine (Cim) (each  $10^{-5}$  M, 1 h), and then incubated with the indicated dose of histamine for 24 h in the presence of the inhibitors. The culture supernatants were assayed for NGF. Values are the mean  $\pm$  SEM of duplicate determinations; \*p<0.05, \*\*p<0.01 vs. control values, determined by one-way ANOVA with Dunnett's post hoc test.

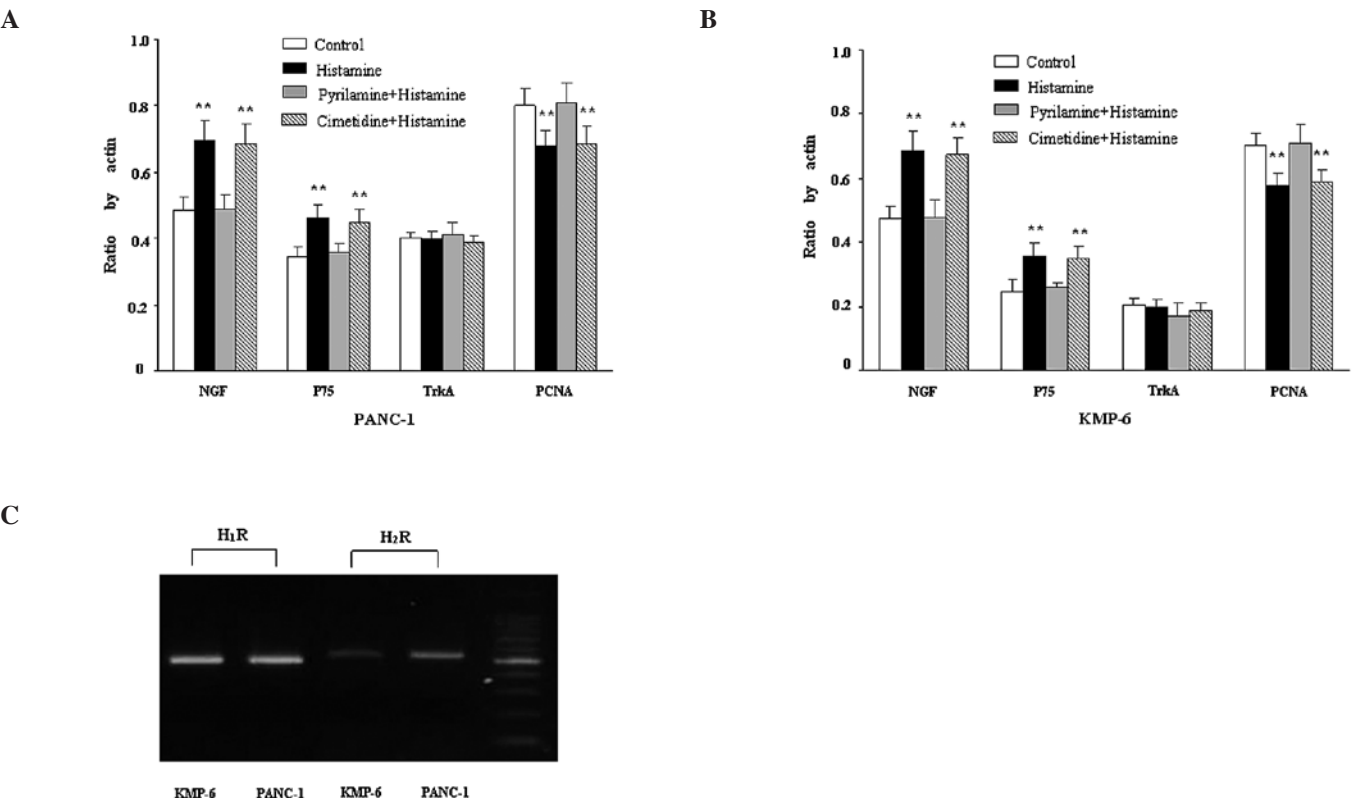


Figure 2. Relative ratio of NGF, P75, TrkA and PCNA mRNA expression in PANC-1 and KMP-6 cells. (A and B) PANC-1 and KMP-6 cells were pre-treated for 1 h with  $10^{-5}$  M pyrilamine or cimetidine and then incubated with  $10^{-6}$  M histamine. mRNA expression was normalized against  $\beta$ -actin as the housekeeping gene. Data is the mean  $\pm$  SEM of duplicate determinations; \*p<0.05, \*\*p<0.01 vs. control values. (C) H<sub>1</sub> and H<sub>2</sub> receptors were identified using RT-PCR as the main receptors in pancreatic cancer cells.



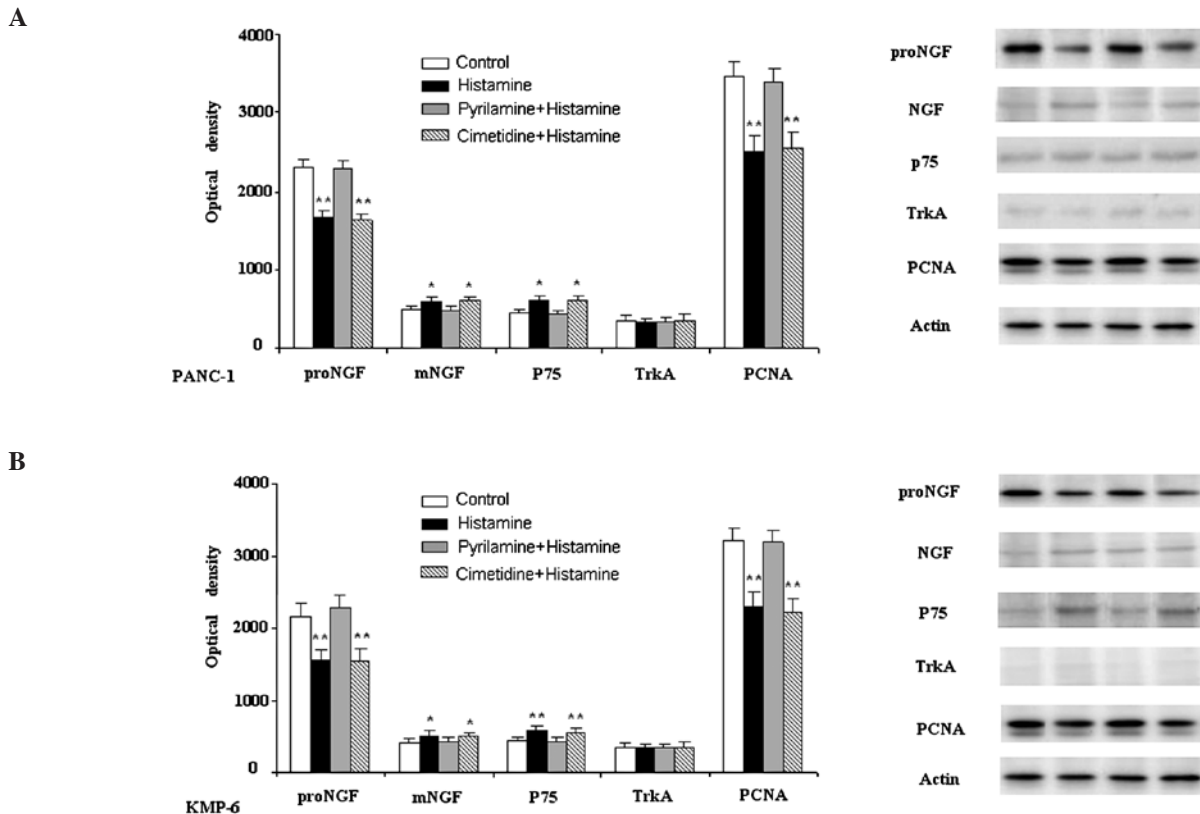


Figure 3. Densitometric analysis and Western blot analysis of NGF ligands, receptors and PCNA. (A and B) Pancreatic cancer cells were pre-treated for 1 h with  $10^{-5}$  M pyrilamine or cimetidine and then incubated with  $10^{-6}$  M histamine. Protein expression was normalized against  $\beta$ -actin. A representative Western blot analysis of these proteins for PANC-1 (A) and KMP-6 (B) is shown. Values are the mean  $\pm$  SEM of duplicate determinations; \* $p<0.05$ , \*\* $p<0.01$  vs. control values.

18.4% in KMP-6,  $p<0.05$ ). Histamine stimulation contributed to an increase in low-affinity receptor p75 protein expression and a decrease in PCNA protein expression. By contrast, no significant changes in TrkA protein expression were observed. The effects of histamine were counteracted by  $H_1$  antagonist pyrilamine, but not by the  $H_2$  antagonist cimetidine.

## Discussion

The results of the present study indicate that histamine is one of the important factors stimulating NGF production and secretion in pancreatic cancer cells. Exposure of pancreatic cells to histamine for 24 h caused alterations in the expression of neurotrophin ligands/receptors (NGF/TrkA and p75). Elevated NGF expression and secretion, but decreased proNGF, were observed in histamine-treated human PANC-1 and KMP-6 cells via  $H_1$  receptor. A correlated increase was observed for p75, while TrkA was not affected. PCNA, which is involved in cell proliferation, was significantly decreased by histamine. Changes in these parameters coincided at the mRNA and protein levels. The observed alterations indicate that histamine has a critical effect on nerve growth factor/receptor expression in pancreatic cancer cells via  $H_1$  receptor-induced signaling.

These results are consistent with those of previous studies on keratinocytes and astrocytes (24-26), and have previously been attributed to the accumulation of mNGF by histamine. However, here Western blot analysis showed that proNGF is the predominant form of NGF in pancreatic cancer cells, and

that mNGF was only faintly detected. Moreover, in striking contrast to an increase in mNGF, intracellular proNGF was down-regulated by histamine.  $H_1$  receptor antagonist pyrilamine was capable of blocking this effect. This contradictory observation may be due to the fact that NGF secretion was quantified by highly sensitive ELISA, which does not discriminate mNGF from the proNGF form. Although the less sensitive Western blot analysis does not allow the accurate investigation of the so-called constitutive release, it allowed us to define with precision the molecular NGF forms involved in the activity-dependent release of this neurotrophic peptide. Meanwhile, the extent of the responsiveness of proNGF and mNGF to histamine exposure relevant to the controls ( $\times 100\%$ ) was profoundly different. In PANC-1 and KMP-6 cells, the responsiveness of intracellular mNGF protein to histamine was approximately 125.06 and 122.56% compared to the control, while that of proNGF was 71.57 and 72.03%, respectively. On the other hand, extracellular NGF measured by ELISA was exponentially increased by histamine stimulus. As previously reported, precursor proNGF is stable and can be converted into the mature intracellular and extracellular forms of NGF (5,30); the possible cause of divergence is that proNGF is cleaved by rapid transportation and/or enzymatic inactivation to mNGF. Histamine, like neuronal stimulation, may induce pancreatic cells to release proNGF, plasminogen, tPA, pro-MMP9 and TIMP-1 into the extracellular space, in turn activating continual cascades and the conversion of plasminogen to plasmin. The generated plasmin may be

capable of converting proNGF into mNGF, which is degraded by activated MMP-9 (5,30,31). Consequently, this discrepancy may reflect a post-translational disturbance in NGF biosynthesis that increases the processing of proNGF to mNGF with histamine stimulation.

The differential effects of histamine exposure on nerve growth factor/receptor (TrkA and p75) expression are suggestive of the mechanisms of responsiveness involved. For example, it is possible that histamine directly up-regulates the number of p75 receptors presenting on PDAC cells by increasing their production, thus decreasing their degradation into the cell membrane. It is also possible that histamine indirectly increases p75 receptors by increasing exogenous NGF, as previously shown (32). The differential effects of histamine on p75 and TrkA possibly elucidate the disruption of cancer cell proliferation and apoptosis (33–38). Our observation that the expression of PCNA in histamine-treated cells was significantly decreased compared to the control cells is consistent with this conclusion, given that histamine has been shown to inhibit proliferation in pancreatic carcinoma cell lines and to modulate apoptotic signaling factors (37,38). We therefore conclude that histamine is likely to modulate pancreatic cancer cell biological function and metabolism by varying the equilibrium between p75 and TrkA. However, the etiological mechanisms of this process have yet to be determined, and further studies are needed to confirm this hypothesis.

Histamine receptors in cancer cells have been extensively characterized in the presence of H<sub>1</sub>–H<sub>4</sub>-receptor binding sites by several authors (38–40). In the current experimental model of human pancreatic cancer cells, we identified the histamine H<sub>1</sub> and H<sub>2</sub> receptor subtypes, and confirmed our initial hypothesis that histamine H<sub>1</sub> receptor subtypes are involved in the signaling pathway of histamine-stimulated NGF secretion. In addition, the H<sub>2</sub>-receptor antagonist cimetidine was not able to diminish the stimulatory effect of histamine on NGF secretion. We therefore could not confirm the involvement of the H<sub>2</sub>-receptor subtype in the enhancement of NGF secretion provoked by histamine. Although H<sub>1</sub>- and H<sub>2</sub>-receptor subtypes were the main types identified in the pancreatic cancer cells, the role of other histamine receptor subtypes cannot be excluded from the regulation of NGF production in other cells. Our data are based on recent studies on human keratinocytes and astrocytes, in which pyrilamine was able to completely inhibit the stimulatory effect of histamine on NGF secretion (24,25). These findings strongly indicate that H<sub>1</sub> receptor subtype is involved in the signal transduction pathway responsible for histamine-stimulated nerve growth factor/receptor expression in pancreatic cancer cells.

In conclusion, we demonstrated that histamine enhances the production of NGF in cultured pancreatic cancer cells and induces alterations in corresponding receptors by stimulating the histamine H<sub>1</sub> receptor. These findings contribute to the clarification of molecular mechanisms involved in the interaction between histamine and nerve growth factors/receptors in cancer cells, which potentially participates in proliferation disorders. We may further conclude that histamine exerts profound effects on the biological response of pancreatic cancer cells through neurotrophins/receptors, and represents a promising target for the development of more specific and less toxic cancer therapies.

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