

The Effect of Apelin on the Functions of Peritoneal Macrophages

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Summary Apelin, the endogenous ligand of the G protein-coupled receptor (APJ), plays an important role in the physiological response to homeostatic perturbations. The aim of

the present study was to investigate the effect of apelin on the functions of peritoneal macrophages. A double staining immunofluorescence technique was used to determine the expression of APJ in peritoneal macrophages. Rat peritoneal macrophages were randomly divided into three groups: control, apelin and apelin+F13A. A significant decrease in phagocytic and chemotactic activity of peritoneal macrophages resulted when the macrophages were incubated with [Pry¹]-Apelin-13 (10 ng/ml). Incubation of peritoneal macrophages with the APJ receptor antagonist, F13A (20 ng/ml) prevented the suppressive effect of apelin on phagocytosis and chemotaxis. Peritoneal macrophages incubated with [Pry¹]-Apelin-13 exhibited a decrease in the production of TNF- α and IL-6 compared to the control macrophages. Incubation of peritoneal macrophages with [Pry¹]-Apelin-13 plus F13A prevented the decrease in the production of proinflammatory cytokines produced by [Pry¹]-Apelin-13. In conclusion, apelin may be a mediator that inhibits the functions of activated macrophages.

Key words: apelin, APJ, macrophages, chemotaxis, phagocytosis

Introduction

Macrophages are professional phagocytes, and they play a central role in the immune response to inflammatory and infectious diseases (Izgut-Uysal *et al.* 2004, Meneguello-Coutinho *et al.* 2014). They appear to be involved in antigen presentation and in the secretion

of agents that stimulate immune cell (Babior 2000). Macrophages also have the capacity to destroy microorganisms and tumor cells through reactive oxygen species formation by the respiratory burst reaction (Ghonime *et al.* 2015). Macrophages are essential cells for immunity, and they are absolutely required to build and modulate the innate immune response. The recognition and subsequent engulfment of apoptotic polymorphonuclear neutrophils by macrophages is a key event in the resolution of inflammation (Kantari *et al.* 2008, Wynn *et al.* 2010).

Apelin is an endogenous ligand of the G protein-coupled receptor APJ (Tatemoto *et al.* 1998). Apelin is initially synthesized as a 77-amino acid prepropeptide that is cleaved into shorter forms. The main active forms of apelin are apelin-13, apelin-17, apelin-36 and the pyroglutamylated isoform of apelin-13, [Pyr¹]-apelin-13 (Pitkin *et al.* 2010). Apelin and APJ are expressed in the brain, kidneys, lungs, mammary glands, cardiovascular system, and gastrointestinal tract (Medhurst *et al.* 2003, Kleinz *et al.* 2005, Carpena *et al.* 2007, Pitkin *et al.* 2010, Gautier *et al.* 2012). Apelin is involved in the regulation of appetite, drinking behavior, blood pressure, heart contractility, pituitary hormone secretion, cell proliferation and apoptosis (Ashley *et al.* 2006, Cox *et al.* 2006, Reaux *et al.* 2001, Taheri *et al.* 2002). Apelin expression is regulated by factors such as hypoxia, stress, fasting and refeeding (Han *et al.* 2008, Izzut-Uysal *et al.* 2014). Additionally, apelin expression is induced by inflammatory mediators such as TNF- α (Daviaud *et al.* 2006). In fact, apelin transcriptional activity stimulated by inflammatory mediators and increased apelin expression during inflammation in intestinal tissue has been shown (Han *et al.* 2008).

It has been reported that apelin reduces the concentrations of IL-6 and MCP-1 protein in the supernatant of cultured J774 macrophages and also inhibits macrophage accumulation in the vessel wall. Furthermore, prior to lipopolysaccharide (LPS) stimulation, pretreatment with [Pyr¹]-apelin-13 significantly diminished mRNA expression and protein secretion of interleukin-6 in J774.1 cells (Obara *et al.* 2014). However, apelin does not modulate the expression of adhesion molecules such as VCAM-1, ICAM-1 and E-selectin which play an important role in leukocyte migration into the inflammatory area (Leeper *et al.* 2009).

Although neural macrophages do not express APJ receptors (Edinger *et al.* 1998, Choe *et al.* 2000, Hashimoto *et al.* 2006), Leeper *et al.* showed that murine monocytes and macrophages express the apelin receptor APJ (Leeper *et al.* 2009), which suggests that macrophages are responsive to apelin through APJ.

The anti-inflammatory effect of apelin down regulating the expression of inflammatory cytokines and chemokines in cultured cells has been shown (Leeper *et al.* 2009). However, the direct effect of apelin on macrophages is not known. The aim of the present study was to investigate the effect of apelin on the functions of peritoneal macrophages.

Materials and Methods*Animals*

Twenty male Wistar rats weighing between 200-250 g were fed a standard laboratory chow diet and tap water ad libitum and maintained in a controlled environment at 22 \pm 1°C with a 12-h dark/light cycle. The study protocol was approved by the Akdeniz University Animal Care and Use Committee.

Experimental Protocols

To determine the effect of apelin on the function of macrophages, peritoneal cells were harvested from the rat peritoneum. Animals were anesthetized with diethyl ether inhalation.

Ten milliliters of Krebs buffer was injected intraperitoneally. After 3 min, the abdomen was opened by a midline incision without hemorrhage, and peritoneal exudate was collected. Peritoneal cells in exudate contained %84 macrophage, %7.2 mast cell and %8.5 eosinophil. The peritoneal cells were separated by centrifugation (1600 rpm for 10 min at 4°C) and the total number of cells was counted by an electronic hematology analyzer (Micros, ABX Co., Montpellier Cedex 4, France). The isolated cells were resuspended with RPMI-1640 and adjusted to 2×10^6 viable cells/ml for the assay of phagocytic activity, % phagocytosis, chemotaxis and the measurement of proinflammatory cytokines. Cell viability was >90%, as determined by the trypan blue exclusion dye test. The peritoneal macrophages obtained from the rats were randomly separated into three test tubes including a control group (n=10), an apelin group (n=10) and an apelin+F13A group (n=10). In the apelin group, [pyr¹]-apelin-13 (10 ng/ml, Tocris Bioscience, Bristol, UK) was added into the tubes containing peritoneal cells (2×10^6 cells/ml) before the incubation period. In the apelin+F13A group, the apelin receptor antagonist, F13A (20 ng/ml, Phoenix Pharmaceuticals, CA, USA) and [Pyr¹]-Apelin-13 (10 ng/ml) were added into the tubes containing peritoneal cells (2×10^6 cells/ml) before the incubation period. All chemical reagents were of analytical grade.

Double Immunofluorescence Staining

To identify the expression of the APJ receptors in the peritoneal macrophages, the following double immunofluorescent procedures were conducted. The smears were prepared from macrophages obtained from the rat peritoneum. A drop of cell suspension was placed on one end of a slide, and by holding another slide at a 45° angle, the cells were dispersed over the slide's length. The slides were left to air dry, and then they were fixed in 3.7% formalin for 20 min. The slides were left to air dry again, and then the slides were either stained or kept at -20°C. To identify the macrophages and apelin receptor expression, we used an immunofluorescence technique. Slides were incubated in 0.5% Triton X-100 phosphate-buffered saline (PBS; pH 7.4) for 10 min to permeabilize the cell membranes. Then, the slides were rinsed in PBS for 3x5 min. The slides were blocked with UV block (Lab Vision Corporation, CA, USA) for 10 min. The slides were then incubated with a mixture of 1:100 anti-macrophage antibody (mouse monoclonal, NeoMarkers, CA, USA) and 1:100 anti-apelin receptor antibody (rabbit polyclonal, Bioss, MA, USA) overnight at +4°C. Negative control staining was performed using normal mouse IgG (Santa Cruz Biotechnology, CA, USA) and normal rabbit IgG (Santa Cruz Biotechnology, CA, USA) separately at the same concentration. The next day, the slides were washed in PBS for 3x5 min. Primary antibody binding was detected using fluorescent conjugated secondary antibodies: for the macrophage antibody and mouse IgG, 1:400, Alexa Fluor 555 donkey anti-mouse IgG (Life Technologies, Eugene, USA); for apelin receptor antibody and rabbit IgG, 1:400 Alexa Fluor 488 goat anti-rabbit IgG (Life Technologies, Eugene, USA) were used for 60 min at room temperature. After incubation in the secondary antibodies, the slides were washed in PBS for 3x5 min. Then, the slides were stained with DAPI (Sigma-Aldrich, MO, USA) for one min, and then they were mounted under Vectashield mounting medium (Vector Laboratories, CA, USA). Photomicrographs were taken with a fluorescent microscope (BX61 microscope, Olympus, Tokyo, Japan) fitted with a DP30BW digital camera.

Measurement of the Phagocytic Activity

One hundred microliters of 1% activated charcoal was added to 100 µL of cell suspension and incubated for 1-h at 37°C. After the incubation period, the number of particles phagocytosed by each macrophage was counted under a light microscope (x1000; Olympus, Tokyo, Japan) to determine the phagocytic activity. Macrophage phagocytic activity was shown as the number of phagocytosed particles per macrophage. % phagocytose refers to the

percentage of cells that had phagocytosed at least one particle. One hundred cells were randomly counted, and the percentage of cells that performed phagocytosis was determined. The phagocytic index is a way to normalize the data relative to the phagocytic activity. The phagocytic index was calculated according to the following equation: % of cells presenting phagocytosis x number of phagocytosed particles/1000).

Measurement of the Chemotactic Activity

Zymosan-activated serum (ZAS, 10%) was prepared according to Goldstein et al. (Goldstein *et al.* 1975). Briefly, rat serum was incubated with boiled and washed zymosan particles at 1 mg/ml for 45 min at 37°C. The zymosan particles were removed by centrifugation (1000 rpm for 10 min) before the supernatant was collected and stored at -20°C in small aliquots until used. For chemotactic activity, the ZAS was diluted 1:10 with incubation medium. The chemotaxis assay was performed by Boyden's method (Chen 2005) using a nitrocellulose filter with an 8 µm pore size (Schleicher and Schuell AE 99 Membrane Filter). A sample of 0.5 ml was prepared to contain 2×10^6 cells per ml with RPMI-1640 medium and added to the upper compartment of the trans-well migration chamber. 0.5 ml of ZAS was injected into the lower compartment of the chamber and incubated for 45 min at 37°C. After the incubation period, the membrane filter was removed, and the cells were stained with hematoxylin. The distance the macrophages migrated (in micrometers) to the lower face of the filter was determined under a light microscope (x1000; Olympus, Tokyo, Japan). Data were expressed as the average of 3 randomly chosen fields (Izgut-Uysal *et al.* 2004).

Measurement of TNF- α and IL-6

A total of 2×10^6 cells were incubated in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) containing 10% fetal bovine serum (FBS; Gibco) for 6 h including 100 ng/ml [Pry¹]-Apelin-13 or [Pry¹]-Apelin-13 plus F13A (20 ng/ml). After centrifugation, TNF- α and IL-6 in cell culture supernatants were measured with enzyme immunoassay kits (Thermo Fisher Scientific, CA, USA) according to the manufacturer's instructions. The results were based on two independent replicates of each sample.

Statistics

Data are expressed as the mean \pm standard error of the mean (SEM). Data analysis was performed with SPSS version 13.0 software (SPSS, Chicago, IL, USA) using the Kruskal-Wallis and Mann-Whitney U tests. Statistical significance was considered to occur at a value of $p < 0.05$.

Results

To further confirm the dose-dependent effect of [Pry¹]-Apelin-13 on macrophage function, one ml of cell suspension containing 2×10^6 cells were exposed to 1, 10 or 20 ng/ml [Pry¹]-Apelin-13 and the phagocytic or chemotactic activity of the macrophages was estimated. For the measurement of macrophage function, the effective dose of [Pry¹]-Apelin-13 was detected to be 10 ng/ml or higher. As seen in Table 1, no significantly different effect was observed between 10 and 20 ng/ml of [Pry¹]-Apelin-13. Thus, in the present study, [Pry¹]-Apelin-13 was used at 10 ng/ml because the suppressive effect on macrophage function was found at this dosage.

APJ Receptor Determination Using Immunofluorescence Staining

To investigate whether apelin has any influence on peritoneal macrophages, we assessed expression of its receptor, APJ in peritoneal macrophages. As shown in Fig. 1, immunofluorescent-stained APJ was expressed by peritoneal macrophages.

The Effect of [Pry¹]-Apelin-13 on the Phagocytic and Chemotactic Activities of Peritoneal Macrophages

As shown in Table 2, the phagocytic capability of peritoneal macrophages was determined by the number of phagocytosed particles per macrophage. The incubation of peritoneal macrophages with [Pry¹]-Apelin-13 decreased the number of phagocytosed particles ($p < 0.01$ compared to the control group, Fig. 2A). The addition of the APJ antagonist, F13A into the incubation medium prevented the suppressive effect of [Pry¹]-Apelin-13 on the phagocytic activity of peritoneal macrophages ($p < 0.01$ compared to the apelin group). Similarly, incubation of peritoneal macrophages with [Pry¹]-Apelin-13 decreased the phagocytic index ($p < 0.01$), and F13A prevented the [Pry¹]-Apelin-13-induced decrease in the phagocytic index. However, neither apelin nor F13A had any effect on the % phagocytosis of peritoneal macrophages.

As shown in Fig. 2B, peritoneal macrophages incubated with [Pry¹]-Apelin-13 presented lower chemotactic capacity compared to the control macrophages ($p < 0.05$). F13A prevented the decrease in chemotactic activity of the peritoneal macrophages by [Pry¹]-Apelin-13 ($p < 0.05$ compared to the apelin group).

The Effect of [Pry¹]-Apelin-13 on the Secretory Functions of Peritoneal Macrophages

To study the effects of apelin on TNF- α and IL-6 secretion by peritoneal macrophages, 10 ng/ml of [Pry¹]-Apelin-13 was added to peritoneal macrophages and incubated for 6 h. Our data shows that proinflammatory cytokines, TNF- α and IL-6 were down-regulated by apelin (Fig. 3). The incubation of peritoneal macrophages with [Pry¹]-Apelin-13 plus APJ receptor antagonist prevented apelin-induced decrease in the proinflammatory cytokine production.

Discussion

Phagocytes are an important component of the immune system in that they engulf and kill the pathogenic microorganisms and in remove damaged and senescent peripheral blood cells, apoptotic and opsonized cells and even inert particles such as latex beads or activated charcoal (Babior 2000, Izgut-Uysal *et al.* 2005). This study was designed to investigate the direct effect of apelin on the functions of peritoneal macrophages. The proposed effects of apelin can appear if the apelin receptor is expressed in macrophages. Despite the studies showing that neural macrophages do not express apelin receptors, Leeper *et al.* (Leeper *et al.* 2009) demonstrated that both murine monocytes and macrophages express APJ receptor mRNA. Our double immunofluorescence staining data seem to support the quantitative RT-PCR data related to the expression of APJ in macrophages as shown by Leeper *et al.* According to our data, peritoneal macrophages express high levels of the APJ receptor, and [Pry¹]-Apelin-13 down regulates the production of proinflammatory cytokines, and the chemotactic and phagocytic activity of peritoneal macrophages. The present study clearly demonstrates for the first time that exogenous apelin may act as a direct regulator of the functions of peritoneal macrophages. Thus, apelin capacity to decrease the production of proinflammatory cytokines, phagocytosis or chemotaxis may largely be explained by its anti-inflammatory effect on macrophages.

In the previous study, plasma level of apelin was determined as 8,38 ng/ml (Antushevich *et al.* 2014) and also, the effective dose of [Pry¹]-Apelin-13 was detected to be 10 ng/ml or higher in the present study. Therefore, we incubated peritoneal macrophages with 10 ng/ml dose of apelin for the measurement of macrophages functions .

The apelin peptide, an endogenous ligand for APJ receptor. Our data obtained by immunofluorescence technique confirmed that peritoneal macrophages express APJ receptors.

As it has been shown in our current study, previous research demonstrated that macrophages express apelin receptor, (Yang *et al.* 2015).

Apelin is a secreted protein that is expressed in various tissues such as the hypothalamus, stomach, kidneys, and mammary glands (Gautier *et al.* 2012, Medhurst *et al.* 2003). The demonstration of apelin release would further support a role for apelin as an endogenous functional peptide for immune cells. The anti-inflammatory effects of apelin have been shown in several studies. Apelin prevents macrophage-induced inflammation in a model of vascular wall inflammation (Leeper *et al.* 2009). In previous studies have been reported that lipopolysaccharide (LPS)-induced production and release of pro-inflammatory cytokines such as TNF- α , IL-6 and IL-8 in rat peritoneal macrophages (Lee *et al.* 2014). In according our findings, apelin down-regulates pro-inflammatory cytokines that are produced by peritoneal macrophages. It is well known that NF- κ B induces expression of genes involved in inflammatory pathways and induces production of inflammatory cytokines (Yang *et al.* 2015). Yang *et al.* demonstrated that the inflammation relevant NF- κ B/JNK signal pathway was inhibited by apelin. Apelin/APJ may be decrease the production of pro-inflammatory cytokines by regulating NF- κ B/JNK signal pathway.

Macrophages are functionally and phenotypically polarized heterogenous population in response to various cytokine signals in tissues. Polarized macrophages are classified as proinflammatory M1 macrophages and anti-inflammatory M2 macrophages (Dimitrijevic *et al.* 2016, Oishi *et al.* 2016, van Stijn *et al.* 2015). The differentiation to M1 phenotype is induced by lipopolysaccharide (LPS) alone or with Th1 cytokines (IFN- γ and TNF- α). M1 macrophages promote inflammation and production of proinflammatory mediators (TNF- α , IL-6, IL-12) and reactive oxygen species. M1 macrophages having anti-bacterial and anti-tumour effects play a role in killing microorganisms in inflammation, but contribute to the destruction of surrounding tissue(Oishi *et al.* 2016). The recent addition to the category of stimuli for M1 macrophages is granulocyte-macrophage-colony stimulating factor (GM-CSF), a cytokine involved in differentiation of macrophages. It has been shown that GM-CSF stimulate macrophage functions including phagocytosis, antigen presentation and proinflammatory cytokine secretion(Dimitrijevic *et al.* 2016). In contrast, anti-inflammatory cytokines, such as IL-4 and IL-13 induce M2 polarization of macrophages. M2 macrophages suppress inflammation and upregulate the production of anti-inflammatory cytokines such as IL-10 and TGF- β (Oishi *et al.* 2016) In the present study, we demonstrated that apelin decreased the production of proinflammatory cytokines (TNF- α and IL-6) and also, suppressed phagocytic and chemotactic activity of peritoneal macrophages. Therefore, our data suggests that apelin may reduce the differentiation to M1 phenotype.Apelin was recently found to diminish macrophage infiltration and reduce MCP-1 and TNF- α mRNA levels in cultured macrophages (Leeper *et al.* 2009). The reduction in macrophage infiltration could be related to the adhesion molecules. However, Leeper *et al.* demonstrated that apelin does not modulate the expression of adhesion molecules such as VCAM-1, ICAM-1, VE-cadherin or E-selectin (Leeper *et al.* 2009). In addition, fewer chemokines were found in the aortas of apelin-treated rats (Sawane *et al.* 2011). These previous findings, suggest that apelin directly inhibits macrophage activity, and our current findings also support the role of [Pry¹]-Apelin-13 in inhibition of macrophage activity. Apelin down regulates chemotaxis, thereby limiting the recruitment of circulating monocytes to the site of inflammation. In the present study, we demonstrated that apelin/APJ has suppressive effect on the functions of peritoneal macrophages. Considering these findings, the therapeutic use of ligands for APJ may be of interest for the treatment of inflammatory diseases. The inhibitory effect of apelin/APJ on macrophages functions may be a reason for apelin's anti-inflammatory effect.

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Declaration of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

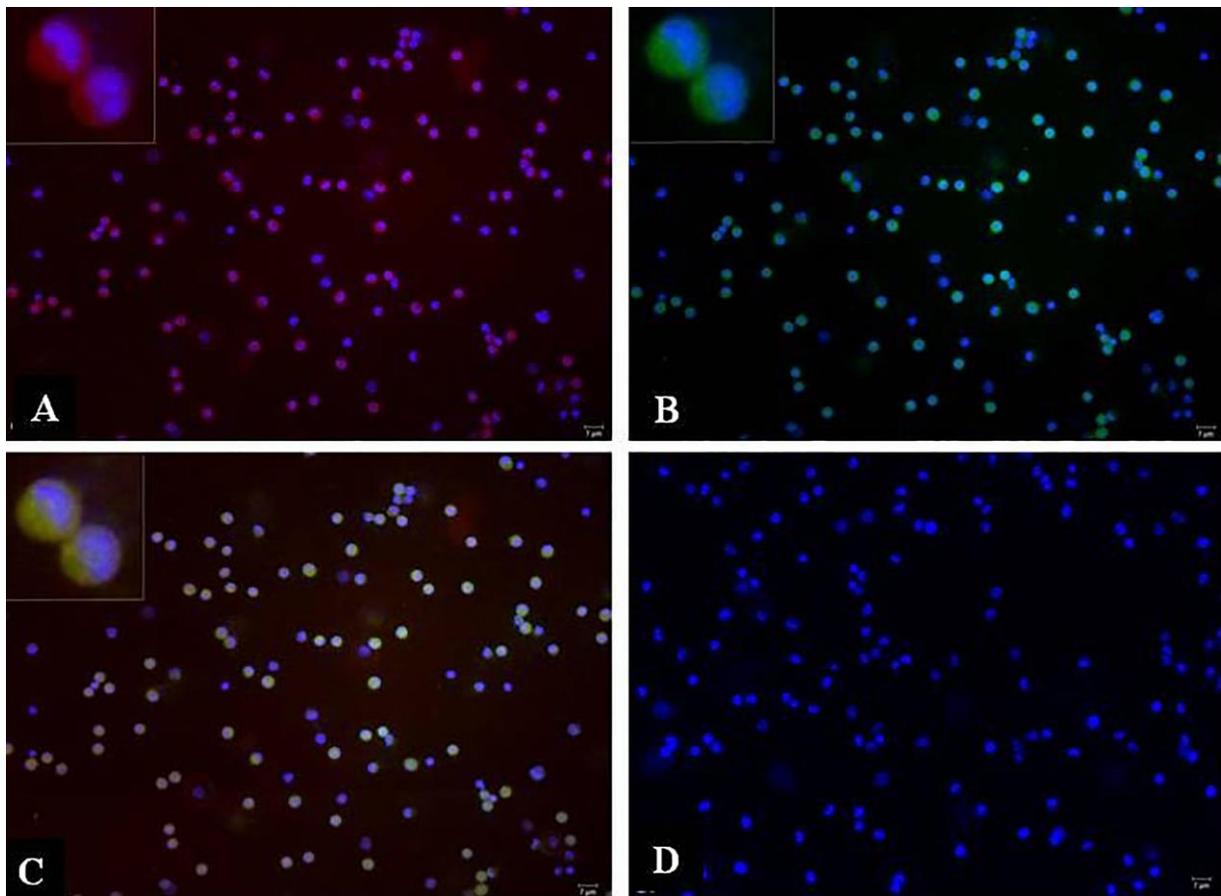


Fig. 1. Double immunofluorescence analysis was performed to determine the APJ receptor expression in peritoneal macrophages. The samples were stained with an anti-macrophage antibody (NeoMarkers, CA, USA) (**A, red**) and an anti-APJ receptor antibody (Bioss, MA, USA) (**B, green**). Signals in panels A, and B were digitally merged in panel **C (yellow)**. Inserts appearing on upper left corners are magnified parts of **A, B** and **C**. A negative control was stained with mouse IgG (Santa Cruz Biotechnology, CA, USA) (**D, blue**). Scale bar=7 μm .

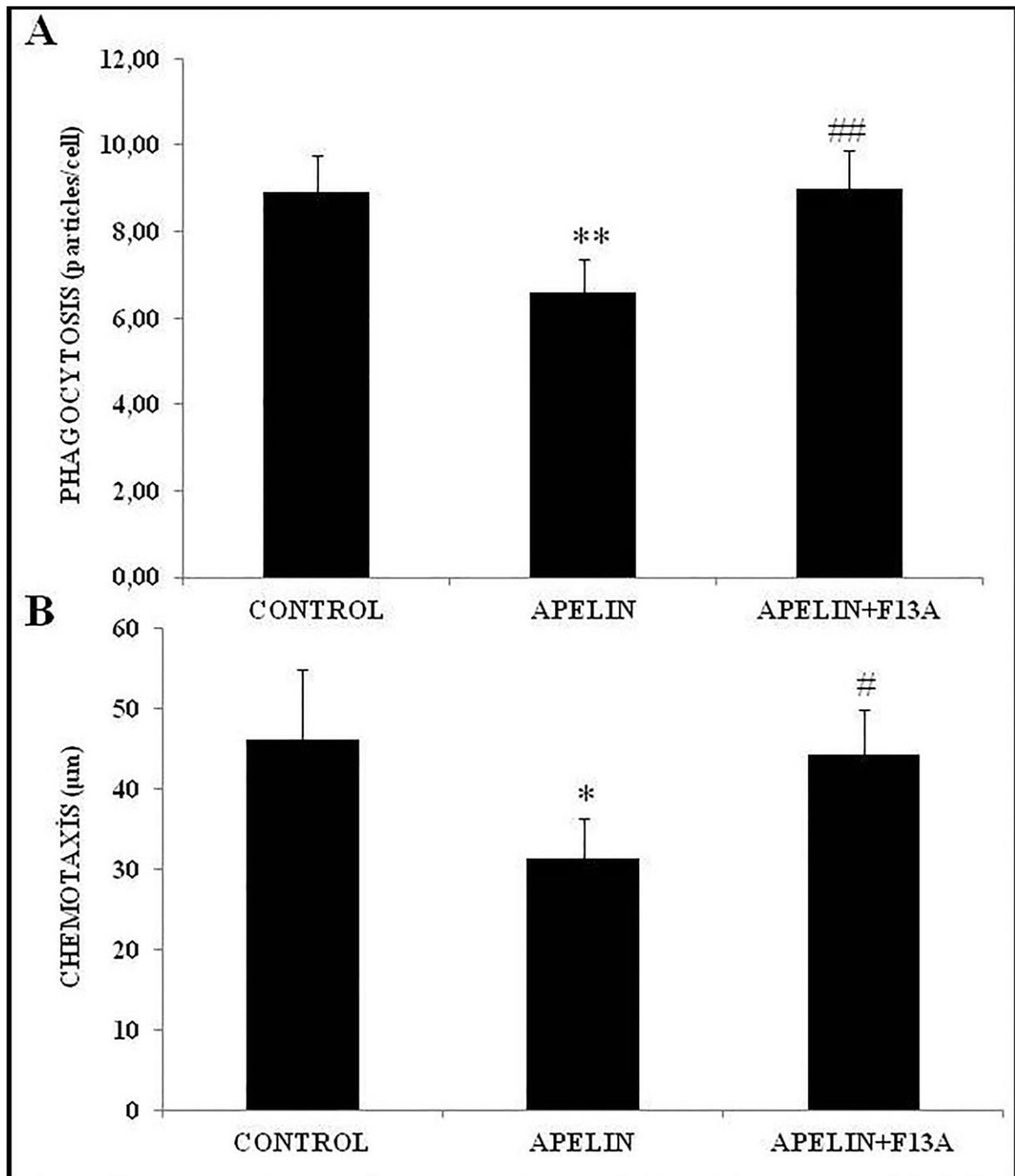


Fig. 2. The effect of [Pry¹]-Apelin-13 and [Pry¹]-Apelin-13 plus F13A on the phagocytic (**A**) and chemotactic (**B**) activities of peritoneal macrophages. **A:** After incubating the peritoneal cells with [Pry¹]-Apelin-13 or [Pry¹]-Apelin-13 plus F13A for 1-h, the number of particles phagocytosed by the macrophages was counted, and averages of one hundred cells were used to determine the phagocytic activity. Data are expressed as the mean±SEM derived from ten experiments performed in duplicate. **p<0.01 compared to the control and ##p<0.01 compared to the apelin group. **B:** The chemotaxis assay was performed using a nitrocellulose filter with an 8 µm pore size. After incubating the peritoneal cells with [Pry¹]-Apelin-13 or [Pry¹]-Apelin-13 plus F13A for 45 min, the membrane filter was stained with hematoxylin. The distance the cells migrated to the lower face of the filter was determined under a light

microscope (x1000; Olympus, Tokyo, Japan). Data are expressed as the mean±SEM derived from ten experiments performed in duplicate. * $p < 0.05$ compared to the control and # $p < 0.05$ compared to the apelin group.

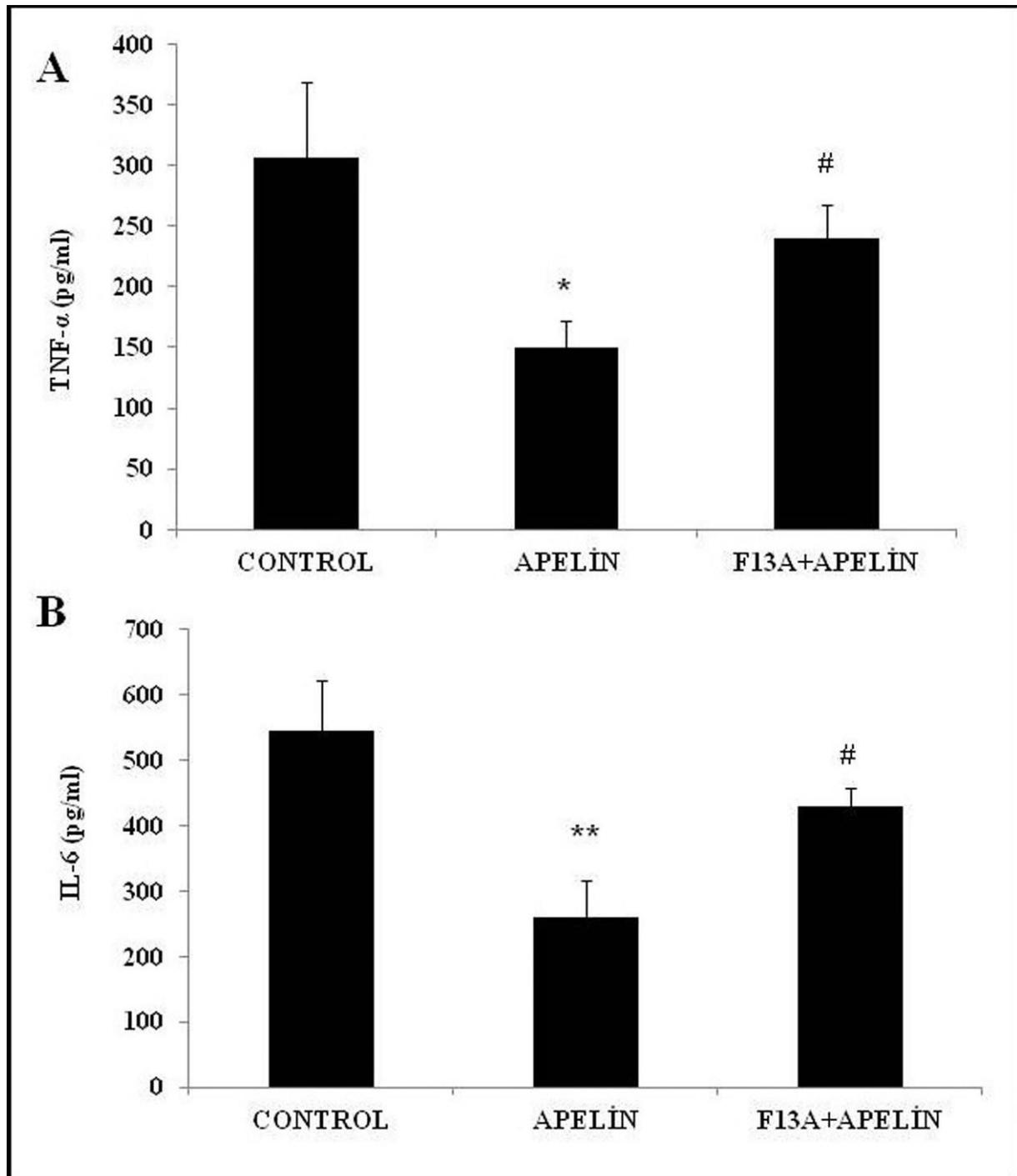


Fig. 3. The effect of [Pry¹]-Apelin-13 and [Pry¹]-Apelin-13 plus F13A on the production of (A) TNF-α and (B) IL-6 by peritoneal macrophages. After incubating the peritoneal cells with [Pry¹]-Apelin-13 or [Pry¹]-Apelin-13 plus F13A for 6 h, TNF-α and IL-6 assay was performed in cell culture supernatants. Data are expressed as the mean±SEM derived from six

experiments performed in duplicate. * $p < 0.05$ and ** $p < 0.01$ compared to the control group, # $p < 0.05$ compared to the apelin group.

Table Legends

	Control	1 ng/mL [Pry ¹]-Apelin-13	10 ng/mL [Pry ¹]-Apelin-13	20 ng/mL [Pry ¹]-Apelin-13
Phagocytic Activity (particles/cell)	8.91±0.85	8.80±0.65	6.60±0.76**	6.90±0.78**
Chemotaxis (µm)	46.10±8.86	49.20±7.65	31.30±5.12*	30.80±4.30*

Data are expressed as the mean±SEM derived from ten experiments performed in duplicate. * $p < 0.05$ and ** $p < 0.01$ as compared with the control group.

Table 1. The dose-dependent effect of [Pry¹]-Apelin-13 on the phagocytic and chemotactic activity of peritoneal cells.

Groups	Phagocytosis %	Phagocytic index
Control	92.00±7.30	0.818±0.013
[Pry ¹]-Apelin-13	90.20±8.50	0.594±0.028**
[Pry ¹]-Apelin-13+F13A	90.35±7.98	0.810±0.016###

Data are expressed as the mean±SEM derived from ten experiments performed in duplicate. ** $p < 0.01$ as compared with the control group, ### $p < 0.01$ as compared with the apelin group.

Table 2. Phagocytosis % and phagocytosis index of peritoneal cells. Phagocytosis index was evaluated according to a equation (% of cells presenting phagocytosis x number of phagocytosed particles/1000) as described in Material and Methods.

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