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Effect of PACAP on Bacterial Adherence and Cytokine Expression in Intestinal Cell Cultures

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Abstract

Bacterial adhesion is a crucial event of intestinal pathological conditions evoked by bacterial infections. Pituitary adenylate cyclase activating polypeptide (PACAP) is an endogenous neuropeptide having a widespread distribution throughout the entire body including the digestive tract. It has diverse physiological functions in the gastrointestinal system, including protective effects in several models of intestinal inflammatory conditions. However, its effects on bacterial adherence and the inflammatory reactions as a result of that have not been elucidated yet. The aim of our study was therefore to investigate the effect of PACAP on bacterial adherence and cytokine expression upon lipopolysaccharide (LPS) exposure. Small intestinal INT407 and colonic Caco-2 cells were treated with PACAP prior to exposure to bacteria (*Escherichia coli, Salmonella* Typhimurium, *Klebsiella pneumoniae, Enterococcus faecalis*) and colonies were counted. PACAP had no significant influence on bacterial adhesion, as it did not change the number of colonies of investigated bacteria. However, PACAP was able to counteract the LPS-induced increases in the expression of the cytokines IL-8 and CXCL-1 in INT407 cells, as assessed by cytokine array. These results indicate that while PACAP has no direct effect on bacterial adherence, it can influence the cytokine expression of intestinal cells upon endotoxin-induced exposure, possibly contributing to the known anti-inflammatory actions of PACAP in the intestinal system.

Keywords Bacterial adherence · Pituitary adenylate cyclase activating polypeptide · Intestinal cell cultures · Lipopolysaccharide · Cytokine expression

Introduction

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a pleiotropic neuropeptide belonging to the secretin/glucagon/vasoactive intestinal peptide family. It was first identified in ovine hypothalamus based on the efficacy on influencing adenylate cyclase activity (Miyata et al. 1989). PACAP has a widespread distribution in the body and exerts a wide range of physiological effects also in the gastrointestinal system (Somogyvari-Vigh and Reglodi 2004; Horvath et al. 2016; Reglodi et al. 2018). The occurrence and functions of PACAP and its receptors can be well demonstrated in neuroendocrine and interstitial cells, in the myenteric and submucosal plexus in the entire length of the gastrointestinal tract and also in the pancreas, gall bladder and liver (Arciszewski et al. 2015; Ji et al. 2013; Koves et al. 1993; Oh et al. 2005; Vu et al. 2016; Zhang et al. 2006; Reglodi et al. 2018). Among others, PACAP influences motility of the intestinal wall (Fujimiya and Inui 2000), inhibits pacemaker activity of interstitial cells of Cajal (Wu et al. 2015) and regulates sphincter function (Farre et al. 2006). PACAP affects release of brain-derived neurotrophic factor in intestinal smooth muscle cells and influences gastric juice secretion (Al-Qudah et al. 2015; Reglodi et al. 2018).

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Several in vivo and in vitro studies confirmed the general cytoprotective, antiapoptotic, antioxidant, and anti-inflammatory effects of PACAP (Reglodi et al. 2012; Ferencz et al. 2009; Horvath et al. 2010; Vaudry et al. 2009). Our previous studies investigating cytoprotective effects have revealed that PACAP is able to exert ambivalent effects on cell viability in the small intestinal INT407 cells depending on the applied stressor and the timing of application (Illes et al. 2017). The anti-inflammatory activity of PACAP can be ascribed to inhibition of immune and inflammatory cells. PACAP decreases the release of inflammatory chemokines and cytokines such as TNF-α and IL-6, inhibits chemotaxis and phagocytosis. Hence, it is an important endogenous immunomodulatory peptide in many different models of inflammatory diseases (Gomariz et al. 2006). In humans, several studies have previously shown changes in PACAP level in colon diseases. An earlier study found significantly lower levels of PACAP in sigmoid colon and rectum tumors compared to normal healthy tissue (Szanto et al. 2012). Another study described significantly higher PACAP levels in patients with symptomatic diverticular disease (Simpson et al. 2009). On the contrary, investigations of colon mucosa of children with ulcerative colitis found decreases in nerve fibers containing PACAP (Kaminska et al. 2006, 2007). Furthermore, our previous investigations have found marked increase in levels of both PACAP isoforms in patients suffering from ulcerative colitis (Horvath et al. 2016). The protective effects of endogenous PACAP can also be detected in the colon: in dextran sulfate sodium-induced colitis the symptoms of the disease in PACAP-deficient mice, such as weight loss, bleeding, diarrhea were markedly more severe than in wild type animals (Azuma et al. 2008). Higher morbidity of colorectal tumors in PACAP-deficient mice indicate the possible regulatory role of endogenously present PACAP (Nemetz et al. 2008). In case of acute ileitis caused by experimental Toxoplasma gondii infection in mice, PACAP prophylaxis improved survival and anti-inflammatory cytokine response (Heimesaat et al. 2014). Besides the beforementioned data, our previous studies have shown differences in microbiota composition in PACAP deficient mice compared to wildtype mice: Bifidobacteria are virtually absent in the knockouts (Heimesaat et al. 2017). A recent study has revealed direct antimicrobial effect of PACAP38 against Gram-positive and Gram-negative bacteria (Starr et al. 2018). All these results indicate that PACAP influences directly and indirectly the intestinal flora and bacterial colonization, which might be a link with increased susceptibility of PACAP deficient mice to intestinal inflammatory diseases and tumors.

Adherence of bacteria to the surface of intestinal epithelial cells is a crucial step in intestinal bacterial infections. Despite the available data on the antimicrobial and intestinal effects of PACAP, its role in bacterial adherence has not been elucidated yet. Therefore, the aim of our study was to explore the role of PACAP in bacterial adhesion in small intestinal INT407 and colon adenocarcinoma Caco-2 cell lines. For this purpose, four different bacteria (*Escherichia coli, Enterococcus faecalis, Klebsiella pneumoniae, Salmonella* Typhimurium) were used. We chose these bacteria because *Escherichia coli, Enterococcus faecalis* and *Klebsiella pneumonia* are commensal bacteria in the gut. Although *Klebsiella pneumonia* is member of the gut flora, it can lead to progression of gastrointestinal diseases such as Crohn's disease and ulcerative colitis (Kaur et al. 2018). Furthermore, *Salmonella* Typhimurium was chosen based on its pathogenic effect in the small intestine.

In addition, our studies were expanded to obtain further information on PACAP's effects on inflammatory processes in the intestinal system using INT 407 cell culture.

Materials and Methods

INT 407 Cell Culture

The INT 407 cell line isolated originally from human embryonic intestinal tissue was purchased from ATCC. INT 407 cells were cultured in Roswell Park Memorial Institute (RPMI 1640) medium (Lonza, Switzerland) supplemented with 10% fetal bovine serum (Biosera, USA) and 1% penicillin–streptomycin (Biosera, USA). Cells were passaged by trypsinization (Trypsin/EDTA; Biosera, USA), followed by dilution in RPMI medium containing 10% fetal bovine serum. Experiments started 24 h after incubation in humified 95% air and 5% CO_2 mixture at 37 °C in the medium.

Caco-2 Cell Culture

The Caco-2 cell line derived from human colon adenocarcinoma cell line was from ATCC. Caco-2 cells were cultured in DMEM high glucose/F-12 supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin (Biosera, USA). Cells were passaged by trypsinization (Trypsin/EDTA; Biosera, USA), followed by dilution in DMEM medium containing 10% fetal bovine serum. Experiments started 24 h after incubation in humified 95% air and 5% CO_2 mixture at 37 °C in the medium.

Determination of Bacterial Adherence to INT 407 and CaCo-2 With and Without PACAP

Before determination of bacterial adhesion, the living INT 407 and Caco-2 cells were counted by trypan blue and 3×10^5 cells/well were plated into 24-well tissue culture plates. Both the small intestinal INT407 cells and the large intestinal Caco-2 cells were grown in their media supplemented with 10% fetal bovine serum without antibiotics in 5% CO_2 at 37 °C. Half of

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the 24-well tissue culture plates contained cells, which were cultured in the above described medium supplemented with 400 ng/ml PACAP. Next day the confluent monolayers were washed three times with 1 ml Dulbecco's Phosphate-Buffered Saline (DPBS) before infection with 3×10^8 bacterial cells in DPBS. After incubation for 3 h at 37 °C in 5% CO_2 each well was washed three times with 1 ml of DPBS. To recover adherent bacterial cells, washed INT 407 and Caco-2 cells in each well were treated with 1 ml of 0.1% Triton-X100 and 0.25% trypsine in PBS for 10 min at room temperature. Each lysate was homogenized by repeated pipetting and 10 µl of their tenfold serial dilutions were plated on Mueller–Hinton agar plates and incubated at 37 °C for 24 h. The following day, the colonies were counted to determine the number of bacteria that had adhered to the small and large intestinal cells. Adherent bacterial counts were obtained from three independent assays with each assay performed in triplicate wells. The investigated bacterial cells were *Escherichia coli* ATCC 25922, *Salmonella* Typhimurium ATCC 14028, *Klebsiella pneumoniae* ATCC 13833, *Enterococcus faecalis* (clinical isolate). Each experiment was repeated six times. Statistical analysis was done using one-way analysis of variance $p < 0.05$ was considered as significant.

Cytokine Array

Investigating the effect of PACAP on cytokine expression Proteome Profiler Human Cytokine Array kit (R&D Systems, Minneapolis, MN, USA) was performed. The investigated INT407 cells were plated in six-well plates and the following experimental groups were created: (1) control group of cells, (2) cells treated with 100 nM PACAP alone for 24 h, (3) cells exposed to 100 ng/ml LPS for 24 h and (4) cells treated with 100 nM PACAP 2 h prior to 24 h-long 100 ng/ml LPS exposion. After incubation, supernatants were collected and were carried out according to the manufacturer's protocol. The kit contains all necessary contents. Briefly, after blocking the membranes for 1 h and adding the reconstituted Detection Antibody Cocktail for another 1 h at room temperature, membranes were incubated with sample/antibody mixture at 2–8 °C overnight. After washing, horseradish peroxidase-conjugated streptavidin was added for 30 min, then membranes were exposed to a chemiluminescent reagent. Array data were analyzed using ImageJ software. The experiment was repeated three times. Statistical analysis analysis was performed by two-way analysis of variance. $p < 0.05$ was considered as significant.

Results

Effect of PACAP on Bacterial Adhesion

To investigate the effect of PACAP on bacterial adhesion PACAP-pretreated and non-pretreated INT407 and Caco-2 cell cultures were infected with the following bacteria: *Escherichia coli* ATCC 25922, *Salmonella* Typhimurium ATCC 14028, *Klebsiella pneumoniae* ATCC 13833 and *Enterococcus faecalis* (clinical isolate). The assay is based on studying the growth of bacteria being able to adhere to cells in vitro. In the current experiment, the influence of PACAP on the number of bacterial colonies adhered to small and large intestinal cells was investigated. PACAP pretreatment of INT407 cells was not able to influence the bacterial adherence (Fig. 1a). Furthermore, similarly to INT407 cells, infections of colon adenocarcinoma-derived Caco-2 cells could not be altered by PACAP-pretreatment (Fig. 1b).

Effect of PACAP on Cytokine Expression in INT407 Cells

To elucidate whether PACAP has an effect on cytokine expression in INT407 cells, we used human cytokine array (Figs. 2, 3). PACAP alone significantly elevated the expression of IL-8 and IL-18 changed slightly the expression of CXCL-1 (C-X-C motif ligand 1) and MIF (macrophage migration inhibitory factor). 100 ng/ml LPS exposure led to higher levels of CXCL-1, IL-8, IL-18 and MIF. These changes were significant in case of IL-8 and IL-18. PACAP-pretreatment was able to attenuate the LPS-induced elevated expression of IL-8 and CXCL-1. Both PACAP and LPS exerted a slight, but not significant, activating effect on MIF.

Discussion

In the present study we found that PACAP could alter the cytokine expression of INT407 small intestinal cells alone and especially after LPS exposure, indicating that the decreased cytokine levels after endotoxin insult can be an additional factor in its anti-inflammatory effect in several intestinal inflammatory conditions. However, we did not find any direct effect on bacterial adhesion, suggesting that PACAP does not affect bacterial adhesion on intestinal cells directly, but is rather involved in the inflammatory reactions induced by different pathogens.

Our finding, that PACAP did not directly influence bacterial adhesion under the applied experimental conditions, is of importance in light of previous findings indicating that PACAP might have direct effects on bacteria and other

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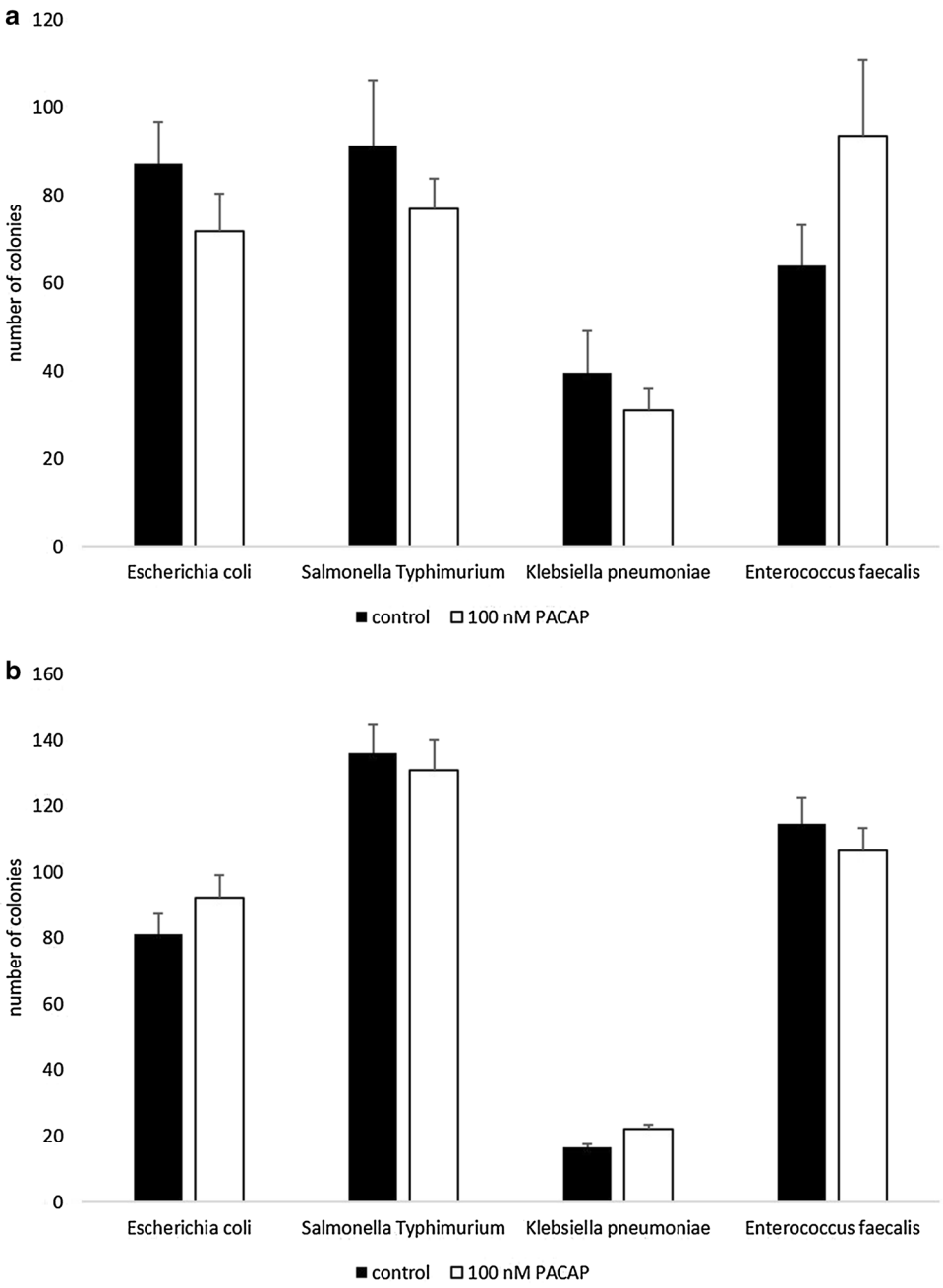

Fig. 1 Effect of PACAP pre-treatment on bacterial adhesion in INT407 (**a**) and Caco-2 (**b**) cells infected with *Escherichia coli, Salmonella* Typhimurium, *Klebsiella pneumoniae* or *Enterococcus faecalis*

pathogens. The first direct anti-microbial effects were proven in Tetrahymena thermophila, a protozoon, where PACAP acted as a chemorepellent (Mace et al. 2000), through the same receptor as lyzozyme (Hassenzahl et al. 2001). A subsequent study found antiparasitic activity against another parasite, *Trypanosoma brucei* (*T. brucei*). Both VIP and PACAP killed the infective bloodstream form but not the noninfective insect form of the parasite (Gonzalez-Rey et al. 2006). Parasite integrity was destroyed through a mechanism involving their entry and accumulation into the cytosol (Gonzalez-Rey et al. 2006). A recent study has proven that PACAP and its related peptides and analogs are able to exert direct antibacterial effects (Starr et al. 2018). Both PACAP38 and 27, as well as related peptides, VIP and secretin, had antibacterial effects against Gram-negative bacteria, such as *Escherichia coli*. PACAP could act against the Gram positive *Staphylococcus aureus*. Another assay showed that PACAP had moderate sterilizing effect against *Pseudomonas aeruginosa* and *Escherichia coli*, an effect less pronounced by the other peptides. PACAP even had a moderate activity against *Bacillus cereus* (Starr et al. 2018). The mechanism of this effect was found to be a membrane permeabilization effect, without causing toxic side effects, as shown by the undisturbed hemolytic activity on red blood cells (Starr

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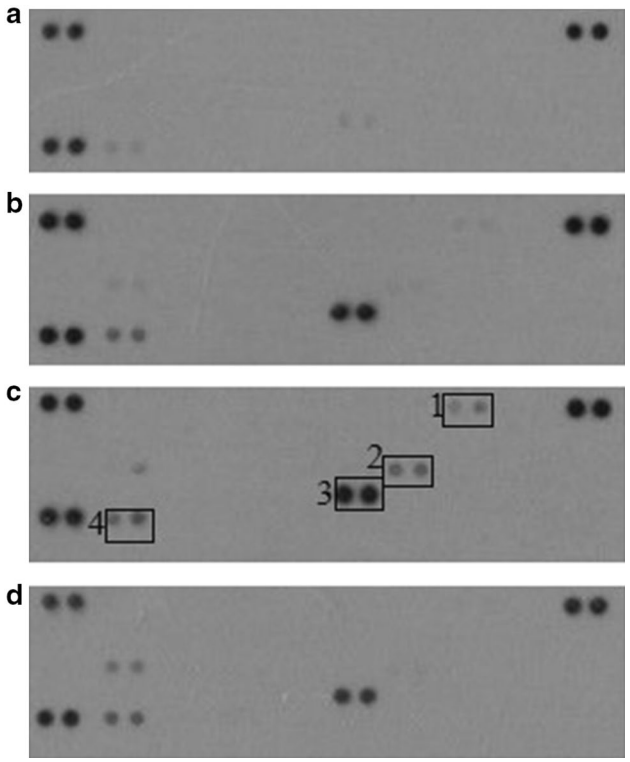

Fig. 2 Representative human cytokine array showing the expression of various cytokines in control INT-407 cells (**a**), cells treated with 100 nM PACAP (**b**), cells exposed to 100 ng/ml LPS (**c**), treatment with 100 nM PACAP 2 h prior to 100 ng/ml LPS stimulation (**d**). (*1*) Changes of CXCL-1 (*1*), IL-8 (*2*), IL-18 (*3*) and MIF (*4*) could be detected. LPS-induced changes of CXCL-1 (*1*) and IL-8 (*2*) were counteracted by PACAP-pretreatment. Other spots, where no significant changes were observed are (from upper left corner, without numbers): CCL-1, CCL-2, MIP-1α, RANTES, CD40 ligand, C5a, CXCL10, CXCL11, CXCL12, G-CSF, GM-CSF, ICAM-1, IFN-γ, IL-1a, IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 p70, IL-13, IL-16, IL-17A, IL-17E, IL-18, IL-21, IL-27, IL-32α, MIF, Serpin E1, TNF-α, TREM-1

et al. 2018). All these data point to the possibility of PACAP acting directly on bacteria. We hypothesized that PACAP might also influence the adhesion of bacteria to the intestinal wall, but we found no effect in the adhesion assay. Therefore, based on our current knowledge, it seems that PACAP can exert a protective effect on bowel inflammatory conditions via both direct antibacterial as well as cytoprotective actions, without influencing the adhesion of bacterial to the intestinal wall.

As a next step, therefore, we investigated the effects of PACAP on cytokine expression of INT407 cells. As PACAP is a known modulator of inflammatory cytokine and chemokine production in various cells, we aimed at testing this effect in small intestinal cells. We found that PACAP altered the expression of several cytokines. IL-8 is a member of the chemokine family identified as a strong chemotactic factor (Baggiolini et al. 1989). Interleukin-8 plays a crucial role in inflammatory, autoimmune and infectious diseases (Harada et al. 1994; Koch et al. 1992; Smyth et al. 1991). In our present study, we detected elevated expressions of IL-8 upon exposure to LPS. PACAP was able to counteract the induction of IL-8 expression. Our finding is in accordance with those of Zhang et al. (2005), who found expression-decreasing effect of PACAP in ARPE cells stimulated with IL-1β. Besides IL-8, we found significantly elevated expression of IL-18 in LPS-induced samples, but in this case no effect of PACAP on it could be observed. Moreover, increase in expression of CXCL-1 could be measured upon LPS exposure. PACAP-pretreatment behaved in an opposite way, it was able to significantly decrease the activation of CXCL-1. Delgado et al. have previously decribed expression-decreasing effect of PACAP in case of LPS-stimulated peritoneal macrophages and microglial cells (2001, 2002). In summary, PACAP is able to alter the expression of several cytokines. This has been demonstrated in many different cell and tissue types, such as lymphocytes (Wang et al. 1999), astrocytes and microglial cells (Gottschall et al. 1994; Delgado et al. 2002), in the retina in a chronic hypoperfusion model (Szabo et al. 2012) and in the kidney, in diabetic and ischemic nephropathy (Horvath et al. 2010; Banki et al. 2013). The effects of PACAP on the cytokine expression varies between cells and also depends on the type of injury. In many cases, PACAP alone does not affect cytokine expression, but can counteract the injury-induced alterations (Szabo et al. 2012). Our observations indicate that while PACAP has no direct action on the bacterial adhesion to the intestinal wall, it can counteract the endotoxin-induced effects on cytokine expression, possibly contributing to the well-known intestinal protective effects of the peptide.

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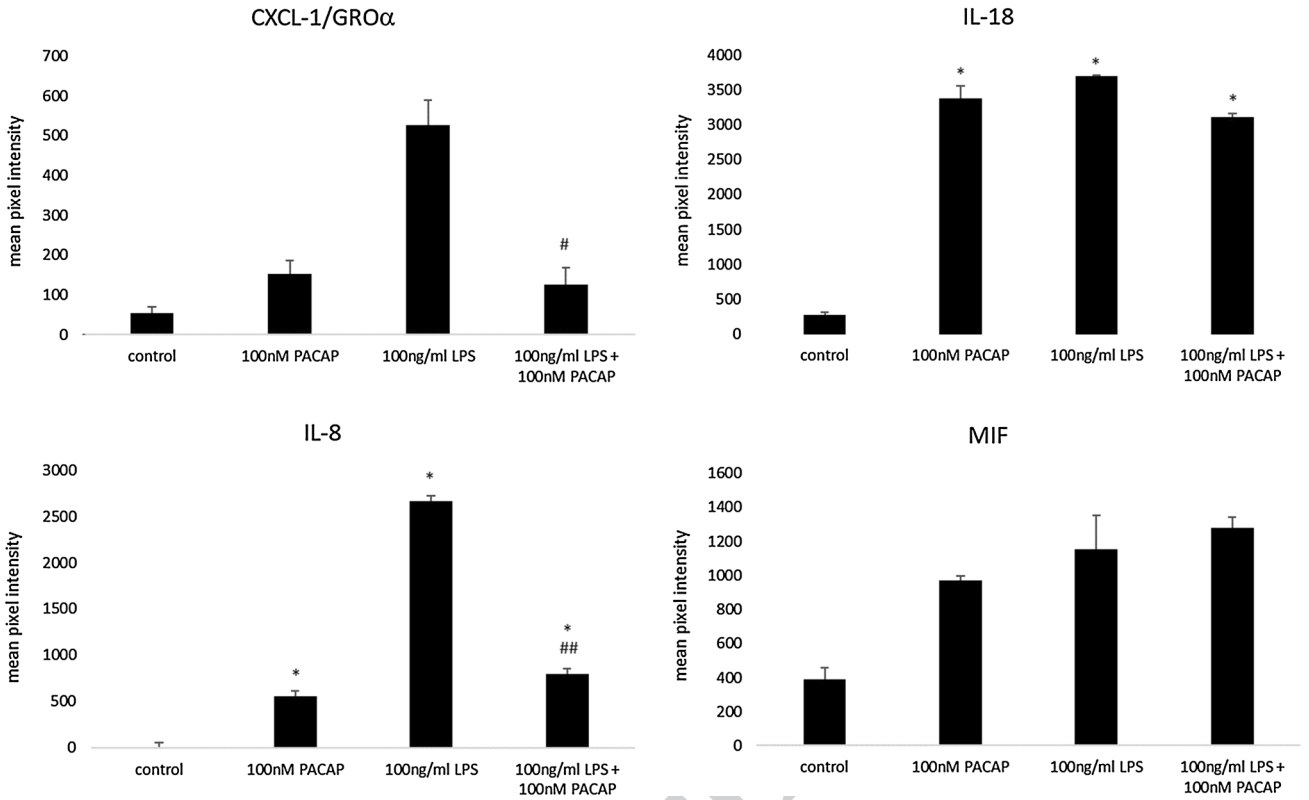

Fig. 3 Quantification of cytokine array. Normalized data are expressed as mean of pixel intensity ± SEM. *$p < 0.05$ versus control group of cells, #$p < 0.05$, ##$p < 0.01$ versus LPS-treated group

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Compliance with Ethical Standards

Conflict of interest All authors declare that they have no conflict of interest.

Research Involving Human and Animal Rights There were neither human nor animal experiments in our studies. Every experiment was done using cell line purchased from ATCC.

Informed Consent Informed consent was not needed because of the in vitro nature of the investigations.

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