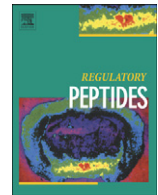
Regulatory Peptides 184 (2013) 22–29

Contents lists available at SciVerse ScienceDirect

Regulatory Peptides

journal homepage: www.elsevier.com/locate/regpep



Calcitonin gene-related peptide promotes the wound healing of human bronchial epithelial cells via PKC and MAPK pathways

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ARTICLE INFO

Article history:
Received 29 March 2012
Received in revised form 21 September 2012
Accepted 3 March 2013
Available online 15 March 2013

Keywords:
Calcitonin gene-related peptide
Wound healing
Human bronchial epithelial cells
Proliferation
Apoptosis

ABSTRACT

Calcitonin gene-related peptide (CGRP) is a 37-amino acid neuropeptide derived from the calcitonin gene. CGRP is widely distributed in the central and peripheral neuronal systems. In the lung, CGRP could modulate dendritic cell function, stimulate proliferation of alveolar epithelial cells and mediate lung injury in mice. In this study, we investigated the effect of CGRP on the wound healing of human bronchial epithelial cells (HBECs) in vitro. The results showed that CGRP accelerated the recovery of wound area of monolayer HBECs in a dose-dependent manner. CGRP inhibited the lipopolysaccharide-induced apoptosis in HBECs. The percentage of S phase and G2/M phase was increased in HBECs after CGRP treatment. CGRP upregulated the expression of Ki67 in a dose-dependent manner. Some pathway inhibitors were used to investigate the signal pathway in which CGRP was involved. We found out that PKC pathway inhibitor (H-7) and MAPK pathway inhibitor (PD98059) could partially attenuate the effect of CGRP, which indicated that CGRP might promote the wound healing of HBECs via PKC and/or MAPK dependent pathway by accelerating migration and proliferation, and inhibiting apoptosis.

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1. Introduction

The airway epithelium acts as a frontline defense against respiratory viruses, not only as physical barrier, but also through the mucociliary apparatus and its immunological function [1]. However, it is susceptible to many respiratory conditions, such as asthma, acute lung injury, and pneumonia [2]. It is reasonable that the repair of the airway epithelium immediately after injury can attenuate the damage and avoid pulmonary disease [3]. During postnatal life, the ability of the lung epithelium to regenerate itself has been well established. Wound healing, restoration of integrity to traumatized tissue, is a normal biological process. In the process of wound healing of airway epithelium, the cells around the wound initially become flat, then disseminate, and migrate toward the wound area. Eventually, basal cells proliferate and differentiate into ciliated epithelium with the recovery of the epithelium [2,4]. Apoptosis also plays an indispensable role in the injury of airway epithelium.

The neuropeptides are widely distributed in neural tissue within the brain, gut, and lung. CGRP is a 37-amino acid peptide derived from the calcitonin gene. It is the most abundant neuropeptide in lung and constitutively expressed in normal lungs where it localizes to pulmonary neuroendocrine cells, a specialized subset of epithelial cells, and sensory C fibers distributed to pulmonary airways [5]. Physiological functions of CGRP are mediated by a family of type II G-protein coupled receptors, and the most important one is the CGRP1 receptor [6]. CGRP was first described as a vasodilator [7]. However, the evidence subsequently suggested that CGRP did not play a pivotal role in the regulation of blood pressure [8]. Recently, many reports clarified the role of CGRP in migraines [9–13]. In the lung, CGRP could modulate dendritic cell function as an anti-inflammatory mediator, and represent a new therapeutic tool in asthma therapy [14]. It also stimulates proliferation of alveolar epithelial cells [15], and mediates acid-induced lung injury in mice [16]. However, it is not clear if CGRP has any effect on wound healing in airway epithelial cells.

In the present study, we focused on the effect of CGRP on wound healing in airway epithelial in vitro. Some pathway inhibitors were used to investigate its possible mechanism.

2. Materials and methods

2.1. Materials

The immortalized HBECs line 16HBE14o — was kindly provided by Dr. Gruenert from the University of California, San Francisco. Dulbecco's modified Eagle's medium (DMEM)/high glucose and newborn calf serum were purchased from Gibco Co. (USA). CGRP, PKC pathway inhibitor (H-7), MAPK pathway inhibitor (PD98059), LPS, propidium iodide (PI), and RNase A were the products of Sigma-Aldrich Co. (St. Louis, MO, USA). The monoclonal antibodies against human Ki67 and the secondary antibody were purchased from Beijing Zhongshan Biotech (Beijing, China). Diaminobenzidine (DAB) histochemistry kit was from Wuhan Boster Biological Technology (Wuhan, China). Annexin V-FITC/PI double

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http://dx.doi.org/10.1016/j.regpep.2013.03.020

staining kit was purchased from Beyotime Institute of Biotechnology (Jiangsu, China).

2.2. Cell culture

HBECs (16HBE14o−) were cultured in the DMED/high glucose containing 100 U/ml penicillin, 100 U/ml streptomycin, and 10% newborn calf serum. Cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2. HBECs were seeded in 12-well plate or 6-well plate according to the experiment.

2.3. Wound-healing assay

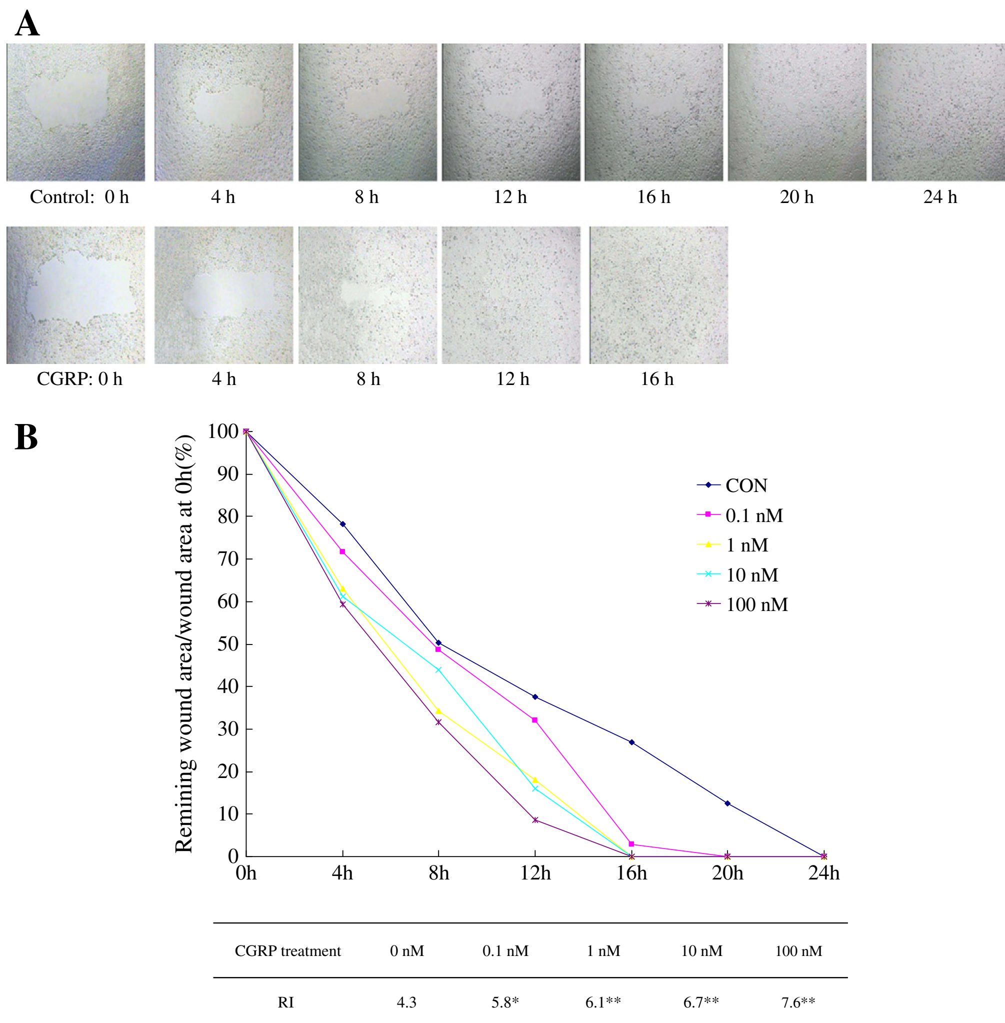
HBECs were seeded in 12-well plate and cultured in the DMED/high glucose. A small wound was generated in the confluent monolayer HBECs by mechanical scraping as described previously [17]. Cells were washed twice with PBS, and medium containing CGRP (0 nM, 0.1 nM, 1 nM, 10 nM and 100 nM) and H-7 (10 μM) or PD98059 (10 μM) was added to the cells. The wound area was observed every 4 h for 24 h by video microscopy (Olympus Company, Japan) and measured by an Image Pro+ System. A linear regression equation of the remaining wound area to time was obtained. Repair index (RI), which is equal to the absolute value of slope, was used to judge the repair rate of HBECs.

2.4. Annexin V-FITC/PI double staining

To determine the impact of CGRP on apoptosis in LPS-treated HBECs, Annexin-V/PI staining was employed according to the manufacturer's instructions. Briefly, harvest the cells after the incubation period and wash in cold PBS at 1000 rpm for 5 min. Re-centrifuge the washed cells, discard the supernatant and resuspend the cells in 100 μL 1 × annexin-binding buffer. Add 5 μL annexin V and 1 μL 100 μg/mL PI working solution to each 100 μL of cell suspension. Incubate the cells at room temperature for 15 min, and add 400 μL 1 × annexin-binding buffers, mix gently, and keep the samples on ice. Then analyze the stained cells by flow cytometry (BECKMAN COULTER, USA) at emission 530 nm and 575 nm using 488 nm excitation.

2.5. Cell cycle analysis

HBECs were seeded in 6-well plates, and treated with CGRP (10 nM) for 24 h. HBECs were trypsinized, centrifuged (1000 rpm, 5 min), washed with cold PBS, and then fixed with cold 70% ethanol at 4 °C overnight. Cells were washed and centrifuged with cold PBS to remove the ethanol. Finally, cells were resuspended with 1 mL PBS, 1000 U RNase A was added, and cells were stained with 1% PI at 4 °C for 30 min. The DNA profiles were determined within 2 h by flow cytometry (BECKMAN COULTER, USA). S-phase fraction (SPF)



CGRP treatment	0 nM	0.1 nM	1 nM	10 nM	100 nM
RI	4.3	5.8*	6.1**	6.7**	7.6**

Fig. 1. CGRP accelerated the recovery of the wound area in a dose-dependent manner in HBECs. A small wound area was generated by mechanical scraping in monolayer HBECs. The area of the wound was observed by microscopy. CGRP treatment (10 nM) could accelerate the recovery of the wound area, which disappeared completely in 16 h compared with 24 h in normal group (A). CGRP promoted the recovery of wound area in HBECs in a dose-dependent manner (B). *$P < 0.05$ vs. control group, **$P < 0.01$ vs. control group.

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