Peptides 32 (2011) 1324–1329

Contents lists available at ScienceDirect

Peptides

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

Neuropeptide Y, an orexigenic hormone, regulates phagocytic activity of lizard splenic phagocytes

Sunil Kumar, Umesh Rai*

Department of Zoology, University of Delhi, Delhi 110007, India

ARTICLE INFO

Article history:
Received 14 February 2011
Received in revised form 10 April 2011
Accepted 11 April 2011
Available online 17 April 2011

Keywords:
Neuropeptide Y
Phagocytes
Reptiles
Signal transduction

ABSTRACT

Present in vitro study in the wall lizard *Hemidactylus flaviviridis*, for the first time in ectothermic vertebrates, demonstrated the immunoregulatory role of neuropeptide Y (NPY) and its receptor-coupled downstream signaling cascade. NPY inhibited the percentage phagocytosis and phagocytic index of splenic phagocytes. The inhibitory effect of NPY on phagocytosis was completely antagonized by Y_2 and Y_5 receptor antagonists. This suggests that NPY mediated its effect on phagocytosis through Y_2 and Y_5 receptors. Further, NPY receptor-coupled downstream signaling cascade for NPY effect on phagocytosis was explored using the inhibitors of adenylate cyclase (SQ 22536) and protein kinase A (H-89). The SQ 22536/H-89 in a concentration-related manner decreased the inhibitory effect of NPY on phagocytosis. Further, an increase in intracellular cAMP level was observed in response to NPY. Taken together, it can be concluded that NPY via Y_2 and Y_5 receptor-coupled AC–cAMP–PKA pathway downregulated the phagocytic activity of lizard splenic phagocytes.

1. Introduction

Neuropeptide Y is a 36 amino acid long peptide, abundantly expressed in central and peripheral nervous system of vertebrates [5,15,16,21,25,31,36,37] and plays an important role as orexigenic hormone in accelerating food intake [35,39] and maintaining glucose homeostasis [17]. Further, to manage the increased energy demand, NPY activates lipogenic enzymes in white adipose tissues and decreases energy expenditure [32]. Thus, it is also being considered as one of the stress hormones. Considering phenomenal increase of NPY expression during stress in different vertebrate species [6,7,9,19,20,26,28,40,41], its inevitable role in immune homeostasis was envisioned. The NPY is shown to have diverse effects on immune responses depending on species [1,3,10], age [11–13,22,30], and concentration [3]. Nonetheless, all the studies so far reported on NPY and immunity are confined to mammals [1–4,10–13,22,30].

Neuropeptide Y, in general, mediates its effect through Y_1, Y_2, Y_4, and Y_5 receptors [23]. The functional existence of these NPY receptors is demonstrated on non-specific immune cells of mammals pharmacologically as well as by cloning [3]. Another receptor, namely y6, is recently reported, though it is non-functional in primates [18]. The NPY is shown to transduce its effect on immune cells through diverse intracellular signaling mechanisms namely cAMP, Ca^{2+}, and PKC pathways [10–12,22]. The present work intends to explore the immunomodulatory role of NPY and its receptor-mediated downstream signaling mechanisms in splenic phagocytes of an ectothermic vertebrate.

Wall lizards, an ectothermic vertebrate, are in intimate contact with their environment that contains high concentration of bacteria and virus. In spite of that they maintain a healthy state by fighting against these invaders with complex system of defense mechanisms, especially innate immune responses. Innate immunity becomes critical for the survival of these ectothermic vertebrates as their adaptive immune system remains compromised in extreme environmental conditions. In view of this, the present in vitro study explores the effect of NPY on phagocytic activity of splenic phagocytes of wall lizards *Hemidactylus flaviviridis* that belongs to reptiles, the group which is phylogenically important being ancestors to both birds and mammals.

2. Materials and methods

2.1. Animals

Adult wall lizards, *H. flaviviridis*, weighing 8–10 g were captured from the suburbs of Delhi, India (Delhi: latitude: 28°12′–28°53′N, longitude: 76°50′–77°23′) and kept in wooden cages having wire mesh at top and sides for proper light and air. Animals were provided food and water *ad libitum*. Prior to experiments, lizards were acclimated to the laboratory conditions for one week (photoperiod

* Corresponding author. Tel.: +91 11 27667443/27667212x106; fax: +91 11 27667985.
E-mail address: rai_u@rediffmail.com (U. Rai).

0196-9781/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.peptides.2011.04.012

from 07:00 to 19:00 h, at room temperature). In the present in vitro study, only female lizards were used due to their better immunological responses [24]. Animal experimental protocols including maintenance and sacrifice of lizards were approved by the Institutional Animal Ethics Committee of the University of Delhi, India.

2.2. Reagents and culture medium

The cell culture medium RPMI 1640, MTT [3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide], adenylate cyclase inhibitor SQ 22536, protein kinase A inhibitor H-89, phosphodiesterase inhibitor 3-isobutyl-1-methyl-xanthine (IBMX), cAMP enzyme immunoassay kit and porcine neuropeptides Y (NPY) were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Y_1 receptor antagonist PD 160170, Y_2 receptor antagonist BIIE 0246 formate, and Y_5 receptor antagonist CGP 71683 hydrochloride were purchased from Tocris Cookson Inc. (Ellsville, MO, USA). The molecular biology grade chemicals of regular use were purchased from Sisco Research Laboratories Pvt. Ltd. (Mumbai, India), Merck Specialties Pvt. Ltd. (Navi Mumbai, India), Central Drug House (P) Ltd. (New Delhi, India) and Qualigens Fine Chemicals (Mumbai, India).

Cell culture medium RPMI 1640 was supplemented with 100 μg/ml streptomycin, 100 IU/ml penicillin, 40 μg/ml gentamicin, 5.94 mg/ml HEPES buffer {N-(2-hydroxyethyl) piperazine-N′-(2-ehtanesulphonic acid)} and 0.2% sodium bicarbonate. In order to prepare the complete culture medium, 2% heat-inactivated fetal calf serum (FCS) (Biological Industries, Kibbutz Beit, Haemek, Israel) was supplemented in culture medium prior to use.

2.3. Preparation of splenic phagocyte monolayer

The method of Mondal and Rai [24] was followed to prepare the lizard splenic phagocyte monolayer. In brief, lizards were sacrificed by cervical dislocation, spleens were dissected out and pooled in chilled phosphate-buffered saline (PBS, pH 7.2). The single cell suspension of splenocytes was made by forcing the spleens through nylon mesh (pore size 90 μm) in ice-cold complete culture medium. Splenocyte number was adjusted to 10^6 cells/ml. To prepare the splenic phagocyte monolayer, 200 μl of splenocyte suspension was flooded on each pre-washed slide. Phagocytes were allowed to adhere for 90 min. Thereafter, non-adherent cells were washed off with PBS. The viability of adhered cell population was above 98% as checked by trypan blue exclusion method. All the experiments were carried out at 25 °C (±1) in humidified chamber/incubator maintained with 5% CO_2.

2.4. Preparation of yeast cell suspension

To prepare the yeast cell suspension, commercially available Baker's yeast (*Saccharomyces cerevisiae*) (1.5 mg/ml PBS) was heated at 80 °C for 20 min. The heat-killed yeast cell suspension was washed and resuspended in the complete culture medium.

2.5. Phagocytic assay

The phagocyte monolayer was incubated with 400 μl yeast cell suspension. After 90 min of incubation, non-phagocytosed yeast cells were washed off with PBS. The monolayer was fixed in methanol, stained with Giemsa and mounted in DPX. Phagocytes engulfing one or more than one yeast cell were considered as positive phagocytes. Without any predetermined sequence or scheme, approximately 200 phagocytes were counted per slide. While counting, experimenter was blind to technical details of slides. The percentage phagocytosis and phagocytic index were calculated using formulae [8]:

(a) $$\text{Percentage phagocytosis} = \frac{\text{number of positive phagocytes}}{100 \quad \text{phagocytes}}.$$

(b) Phagocytic index = average number of yeast cells engulfed by each positive phagocytes × percentage phagocytosis.

2.6. In vitro experiments

2.6.1. Effect of neuropeptide Y

Splenic phagocytes were incubated with varying concentrations of NPY i.e., 10^{-12}, 10^{-11}, 10^{-10}, 10^{-9} and 10^{-8} M for 1 h. Those incubated without NPY (in medium alone) for the same duration were considered as control. After treatment, monolayer was washed and processed for phagocytic assay. The concentrations of NPY used in present in vitro study were determined based on the literature available in other vertebrates [3,11–13,22], as no report is available in reptiles. The optimum time period required for NPY effect on phagocytosis was decided based on pilot experiments.

2.6.2. Effect of $Y_1/Y_2/Y_5$ receptor antagonists

Based on concentration-related effect of NPY on phagocytosis, splenic phagocytes were incubated with the most effective concentration of NPY (10^{-10} M) and its ten times higher concentration (i.e., 10^{-9} M) of Y_1 receptor antagonist (PD 160170)/Y_2 receptor antagonist (BIIE 0246)/Y_5 receptor antagonist (CGP 71683), simultaneously, for 1 h. For controls, phagocytes were incubated in medium alone or with the respective concentration of NPY/PD 160170/BIIE 0246/CGP 71683 for 1 h. The concentration of various NPY receptor antagonists was determined based on literature available [3,11,12,14] and pilot experiments conducted using lizard splenic phagocytes.

2.6.3. Effect of inhibitors for adenylate cyclase and protein kinase A

Splenic phagocytes were pre-treated with different concentrations of adenylate cyclase inhibitor SQ 22536 (2.5, 5.0, 7.5, and 10.0 nM)/protein kinase inhibitor H-89 (25, 50, 75, and 100 nM) for 30 min. Thereafter, cells were incubated with 10^{-10} M NPY and varying concentrations of SQ 22536/H-89 for 1 h, simultaneously. For controls, phagocytes were: (a) incubated in medium alone for 90 min, (b) pre-incubated in medium alone for 30 min and then with 10^{-10} M NPY for 1 h, and (c) incubated with 10 nM SQ 22536/100 nM H-89 for 90 min. After treatment, cells were washed and processed for phagocytic assay. The concentration range of the SQ 22536 and H-89 was determined based on the literature available [33,34] and pilot experiments.

2.7. cAMP assay

The intracellular cAMP content was estimated in NPY-treated splenic phagocytes following the manufacturer's protocol (Sigma–Aldrich, St. Louis, MO, USA). In brief, 100 μl splenocyte suspension was added to each well of 96-well tissue culture plate. After 90 min incubation, non-adherent cells were washed off using PBS (pH 7.2). The adhered cells were incubated with phosphodiesterase inhibitor, IBMX (10^{-4} M) for 30 min. Thereafter, phagocytes were incubated with PBS alone (control) or 10^{-10} M NPY for 1 h. Cells were washed and lyzed with 0.1 M HCl. The cell lysate was centrifuged at 600 *g* for 10 min at room temperature. Cell debris was discarded and, intracellular cAMP content was estimated in cell lysate using cAMP enzyme immunoassay kit.