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β-Endorphin inhibits phagocytic activity of lizard splenic phagocytes through μ receptor-coupled adenylate cyclase-protein kinase A signaling pathway

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ABSTRACT

The receptor-coupled intracellular signaling mechanism of endogenous opioid peptide β-endorphin (β-end) is explored for the first time in ectothermic vertebrates using wall lizard as a model. β-End inhibited the percentage phagocytosis and phagocytic index of lizard splenic phagocytes in a dose-dependent manner. The inhibitory effect of β-end on phagocytosis was completely antagonized by non-selective opioid receptor antagonist naltrexone and also by selective μ-receptor antagonist CTAP. However, selective antagonists for other opioid receptors like NTI for δ-receptor and NorBNI for κ-receptor did not alter the effect of β-end on phagocytosis. This suggests that β-end mediated its inhibitory effect on phagocytic activity of splenic phagocytes exclusively through μ opioid receptors. The μ opioid receptor-coupled downstream signaling cascade was subsequently explored using inhibitors of adenylate cyclase (SQ 22536) and protein kinase A (H-89). Both SQ 22536 and H-89 abolished the inhibitory effect of β-end on phagocytosis in a concentration-related manner. Implication of cAMP as second messenger was corroborated by cAMP assay where an increase in intracellular cAMP level was observed in response to β-end treatment. It can be concluded that β-end downregulated the phagocytic activity of lizard splenic phagocytes through μ opioid receptor-coupled adenylate cyclase-cAMP-protein kinase A pathway.

1. Introduction

Beta-endorphin (β-end) encoded by proopiomelanocortin (POMC) gene is expressed primarily in pars distalis and pars intermedia of the pituitary gland. The different forms of β-end are reported in jawed vertebrates due to differential post-translational processing of the same POMC gene product depending on animals and tissue specificity. β-End (1–31) is yielded as an end-product in the anterior pituitary (pars distalis) while its moderate form (1–26) is also present in the intermediate pituitary (pars intermedia) of mammals. Further, an extensive N-terminal acetylation of β-end occurs in the intermediate pituitary cells. In anuran amphibian, *N*-acetylated small form of β-end (1–8) is shown as a major end-product in the intermediate pituitary cells [17,22,23,37,61,66]. The small form of β-end is also demonstrated in other lower ectothermic vertebrates [14,19–21,31,34,64]. However, such small form of β-end is not detected in the pituitary of mammals [24,35], birds [42] and reptiles [16,18]. It is noteworthy that among the reptiles, *N*-acetylated form of β-end is present in turtles [18] but reported absent in lizard *Anolis carolinensis* [18]. In light of this, non-acetylated form of β-end was used in the present study.

There is growing evidence that β-end secretion in the pituitary increases during stress in fishes [62,65], amphibians [40] and mammals [7,8,10,72]. However, studies on physiological significance of β-end in regulation of immune responses which is highly compromised during stress are limited to fishes [56,68] and mammals [11,43,44,60]. Since reptiles occupy a phylogenic pivotal position being ancestor to both classes of endotherms, birds and mammals, the present study in wall lizards was aimed to provide an insight to develop a comparative understanding on immunomodulatory role of non-acetylated form of β-endorphin.

The innate immune system in vertebrates forms the first line of host defense against invading pathogens. In ectothermic vertebrates including reptiles, the innate immune system plays crucial role in eliminating microbial organisms, as adaptive immune system is sluggish and takes time to respond against the pathogens [25]. Phagocytes, importantly macrophages and neutrophils, constitute the major cellular arms of innate immunity. These cells eliminate the pathogens via phagocytosis and also by releasing cytotoxic substances and proinflammatory cytokines. Thus, the present *in vitro* study in the wall lizard is focused on the role of β-end in regulation of phagocytosis.

Although opioid receptors are cloned from immune as well as non-immune tissues of fishes [1,5,6,12,15,46] and mammals [26,32,53,54,70], the functional existence of opioid receptors especially on immune cells is not demonstrated in reptiles. Similarly,

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the second messenger system involved in transducing the effect of opioid peptides intracellularly is yet to be explored in immune as well as non-immune cells of ectothermic vertebrates. Therefore, this study was undertaken to gain an insight about the existence of functional opioid receptors on lizard splenic phagocytes, also, an attempt was made to understand the specific opioid receptor-coupled downstream signaling mechanism involved in transducing the β-end effect on phagocytosis.

2. Materials and methods

2.1. Animals

The Indian wall lizard *Hemidactylus flaviviridis* weighing 8–10 g were captured from the vicinity of Delhi, India (Delhi: latitude: 28°12′–28°53′ N, longitude: 76°50′–77°23′). They were maintained at room temperature in wooden cages encrusted with wire mesh from top, sides and front for the proper aeration and light. Prior to experiments, lizards were acclimated to the laboratory conditions for a week (12L:12D, lights on at 07:00 h). Food and water were provided *ad libitum*. In the present study, only female lizards were used owing to their greater immunological responses [39]. The guidelines of the Institutional Animal Ethics Committee were followed for maintenance and sacrifice of the animals.

2.2. Reagents and culture medium

Molecular biology grade regular use laboratory chemicals 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). The cell culture medium RPMI 1640, non-selective opioid receptor blocker, naltrexone (Nalt), adenylate cyclase inhibitor SQ 22536, protein kinase A inhibitor H-89, phosphodiesterase inhibitor 3-isobutyl-1-methyl-xanthine (IBMX), cAMP enzyme immunoassay kit and MTT [3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide] were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Human β-endorphin (β-end) (NeoMPS, Inc., San Diego, CA, USA) and the selective opioid receptor antagonists like μ-antagonist CTAP (H-D-Phe-Cys-Tyr-Arg-Thr-Pen-Thr-NH_2), $\delta_{1,2}$-antagonist naltrindole hydrochloride (NTI) and κ-antagonist nor-Binaltorphimine dihydrochloride (NorBNI) were the kind gifts from National Institute on Drug Abuse (NIDA), National Institute of Health (Bethesda, USA). Since β-end of reptilian source is commercially unavailable, in this study β-end of human origin was used. Moreover, human β-end is reported physiologically active in lizard *Podarcis sicula* Raf [13].

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. Fetal calf serum (FCS) (Biological Industries, Kibbutz Beit, Haemek, Israel) was heat-inactivated (56 °C for 20 min) and supplemented 2% in culture medium prior to use. Thereafter, culture medium was referred as complete culture medium.

2.3. Isolation of splenic phagocytes

The splenic phagocyte monolayer was prepared following the method of Mondal and Rai [39]. In brief, lizards were sacrificed, spleens were excised and pooled in chilled phosphate-buffered saline (PBS, pH 7.2). Single cell suspension of splenocytes was prepared by forcing the spleens through nylon mesh (pore size 90 μm) in ice-cold complete culture medium. The cell number was adjusted to 10^6 cells/ml. For phagocytic assay, 200 μl of splenocyte suspension was flooded on each pre-washed slide. Phagocytes were allowed to adhere for 90 min. Non-adhered 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

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

2.5. Phagocytic assay

Four hundred microlitre yeast cell suspension was flooded on each slide with adhered phagocyte monolayer. After incubation of phagocytes with yeast cells for 90 min, non-phagocytosed yeast cells were washed off. The monolayer was fixed in methanol, stained with Giemsa and was mounted in DPX. Phagocyte engulfing one or more than one yeast cell was considered as positive phagocyte. Without any predetermined sequence or scheme, approximately 200 phagocytes/slide were counted. The experimenter was blind to technical details of slides while counting. The percentage phagocytosis and phagocytic index were calculated using formulae [9]:

(a) Percentage phagocytosis = number of positive phagocytes/100 phagocytes.
(b) Phagocytic index = average number of yeast cells engulfed by each positive phagocytes × percentage phagocytosis.

2.6. Microscopy

The bright field photomicrographs of splenic phagocytes incubated with medium alone (control), and optimally effective concentration of β-end (10^{-9} M) were captured using 'Nikon Eclipse E400 microscope' attached with 'Nikon Digital Sight-Fi1 camera'.

2.7. In vitro experiments

2.7.1. Effect of β-endorphin

Splenic phagocytes were treated with 10^{-12}, 10^{-11}, 10^{-10}, 10^{-9} and 10^{-8} M concentration of β-end for 1 h. After treatment, cells were washed and processed for phagocytic assay. For control, phagocytes incubated in medium alone for 1 h. The concentrations of β-end for this experiment were determined based on the plasma concentration of β-end reported in different vertebrate groups [38,48,65,73]. Pilot experiment was performed to optimize incubation time for β-end effect on phagocytosis.

2.7.2. Effect of non-selective opioid receptor blocker

Based on our observations on concentration-related effect of β-end, phagocytes were incubated with the most effective concentration of β-end (10^{-9} M) and a non-selective opioid receptor antagonist Nalt (10^{-8} M), simultaneously, for 1 h. Afterward, cells were washed with PBS, and processed for phagocytic assay. To compare the results following control groups were made: (a) phagocytes incubated in medium, (b) phagocytes incubated with Nalt (10^{-8} M), and (c) phagocytes incubated with β-end (10^{-9} M) for 1 h.