

Role of adrenoceptor-coupled second messenger system in sympatho-adrenomedullary modulation of splenic macrophage functions in live fish *Channa punctatus*

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Received 1 November 2006; revised 13 March 2007; accepted 7 May 2007

Available online 13 May 2007

Abstract

In order to understand the role of sympatho-adrenomedullary (SAM) system in mediating stress effect on non-specific immune responses in fishes, the splenic macrophage phagocytic and respiratory burst activities of normal and chemically sympathectomized *Channa punctatus* under restraint stress were studied. Chemical sympathectomy abrogated the differential effects of acute stress on diverse functions of macrophages. The SAM regulation of macrophage activities was substantiated by *in vitro* experiments with catecholamines, the end product of SAM system. Further, for the first time in fishes, different adrenoceptors and their precise second messenger system regulating diverse functions of macrophages by catecholamines were demonstrated. Norepinephrine (NE)/epinephrine (E) decreased the phagocytosis through β -adrenergic receptor as only propranolol, the β -adrenergic receptor antagonist, blocked the suppressive effect of NE/E. However, dopamine (DA) regulates phagocytosis solely via the dopaminergic receptor. The DA effect was mimicked by DA receptor agonists, apomorphine and bromocryptine. Adenylate cyclase system linked to β -adrenoceptor/dopaminergic receptor seems to be involved in mediating the effect of catecholamine on phagocytosis since db cAMP inhibited the phagocytosis in a dose-dependent manner. In case of superoxide production, only phenoxybenzamine, an α -adrenergic receptor antagonist, was seen effective in blocking the stimulatory effect of NE/E. Further, Ca^{2+} as second messenger system coupled to α 1-adrenergic receptor was shown to mediate this effect since phospholipase C (PLC) inhibitor, U73122 and intracellular calcium chelating agent, BAPTA-AM downregulated the NE/E-induced superoxide production. The role of calcium in modulation of superoxide production was also emphasized using calcium ionophore A23187.

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Keywords: Fish; Macrophages; Catecholamines; Adrenoceptors; Second messenger

1. Introduction

Fishes are often exposed to both cognitive and non-cognitive stress in intensive aquaculture. Regardless of stressors, activation of hypothalamic-pituitary-interrenal axis results in increase of corticosteroid production that suppresses immune responses and consequently, increases the susceptibility to infection (Bonga, 1997). Besides, the sympatho-adrenomedullary (SAM) system is another important

component that is involved in mediating stress effect on immune responses, though the reports are limited and the results are contradictory (Narnaware and Woo, 1999). In mammals, catecholamines are the end products of SAM system and have a plethora of effects on immune responses based on the tissue studied, receptors involved, different second messengers employed by them and also the activation state of the immune cells. The catecholamines transduce their biological information either through adrenergic or dopaminergic receptors. Generally, β -adrenergic receptors and D_1 dopaminergic receptors are linked to adenylate cyclase which upon activation increases the intracellular

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cAMP levels. On the other hand, occupancy of α 1-adrenergic receptor leads to stimulation of phospholipase C activity and consequently, increase of cytosolic IP₃ that releases calcium ions from the endoplasmic reticulum. Moreover, the same catecholaminergic receptor has been reported to be coupled with multiple second messenger system (Andersen et al., 1990; Elenkov et al., 2000). By comparison to mammals, the body of information on the role of sympatho-adrenomedullary system in regulation of immune responses in non-mammalian vertebrates, especially in ectotherms is rudimentary and needs to be expanded.

The present study was undertaken to assess the role of SAM system in regulation of splenic macrophage functions in live fish *Channa punctatus*. Spleen is a secondary immune organ dedicated to screening and eliminating blood-borne pathogens and has been reported to be richly innervated with adrenergic nerve fibres (Flory, 1989). Hence, the phagocytic and cytotoxic activities of splenic macrophages under normal and stressed conditions were studied by temporarily ablating the sympathetic nerve fibres using 6-hydroxydopamine, an agent with high selectivity for peripheral noradrenergic nerves. Further, *in vitro* effect of catecholamines on macrophage activities were studied to get the direct evidence pertaining to role of SAM system in control of immune responses in fishes. In addition, an attempt has been made to delineate the pathway of action of catecholamines in splenic macrophages.

2. Materials and methods

2.1. Animals

In the present study, adult female fish (*Channa punctatus*) weighing 80–100 g were procured from neighbouring state of Delhi (latitude 28.38N, longitude 77.2E), acclimated to the laboratory conditions for a fortnight under 12 L:12 D light regimen at $25 \pm 2^\circ\text{C}$, and fed on alternate day with beef liver.

The guidelines of the “Committee for the purpose of control and supervision of experiments on animals” (CPCSEA), Ministry of Statistics and Programme Implementation, Government of India were followed in maintenance and sacrifice of animals.

2.2. Reagents and culture medium

Tissue culture medium RPMI-1640 (Sigma Chemicals, St. Louis, MO) was supplemented with 40 $\mu\text{g}/\text{ml}$ of antibiotic gentamicin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 100 IU/ml penicillin, 5.94 mg/ml Hepes buffer (Sisco Research Laboratories, Pvt. Ltd., India), and 1% heat-inactivated fetal calf serum (FCS; Biological Industries, Beth Haemek, Israel) and is referred to as complete culture medium.

Catecholamines-epinephrine hydrochloride (E), norepinephrine hydrochloride (NE) and dopamine hydrochloride (DA), $N^6,2'$ -*O*-dibutyryladenosine 3',5'-cyclic monophosphate sodium salt (db cAMP), 2-bromo- α -ergocryptine methanesulfonate salt, apomorphine hydrochloride, propranolol hydrochloride, phenoxybenzamine hydrochloride, calcium ionophore A23187, 1,2-bis-(2-aminophenoxy)ethane- N,N,N',N' -tetraacetic acid tetrakis (acetoxy-methylester) (BAPTA-AM), 1-[6-(((17 β)-3-methoxyestra-1,3,5[10]-trien-17-yl)amino)hexyl]-1H-pyrrole-2,5-dione (U73122), nitroterazolium blue chloride (NBT), phorbol 12-myristate 13-acetate (PMA) and 6-hydroxydopamine HBr (6-OHDA) were purchased from Sigma Chemicals (St. Louis, MO). 2-phenoxyethanol, dimethyl sulphoxide (DMSO), methanol, Triton X-100, Giemsa stain and other routine chem-

icals were purchased from Sisco Research Laboratories (SRL) Pvt. Ltd., India/Merck Ltd, India. Stock solutions of PMA, U73122, BAPTA-AM and A23187 were made in DMSO. Further dilutions were made in phosphate buffered-saline (PBS, 1 $\times$, pH 7.8). DMSO was also added to the culture medium used for their respective controls.

2.3. Preparation of macrophage monolayer

The fishes were sacrificed by a lethal dose of 2-phenoxyethanol (1:1000), spleens were dissected out, and forced through a nylon mesh of pore size 90 μm into chilled PBS (1 $\times$, pH 7.8). The cell suspension was centrifuged at 600g for 15 min. The supernatant was discarded. Red blood corpuscles (RBCs) were removed by water shock treatment following the method of Paltrinieri et al. (2002) with minor modifications. Briefly, splenic cell pellet was suspended in 9 ml of distilled water and kept for 30 s to lyse the RBCs. Thereafter, 1 ml of 10 $\times$ PBS (pH 7.8) was added to maintain the isotonicity. The RBC cell debris was removed by washing. Finally, the splenic cell pellet was resuspended in complete culture medium. The cell suspension was flooded on pre-washed slides (200 $\mu\text{l}/\text{slide}$) or added to 96-well culture plate (100 $\mu\text{l}/\text{well}$). Macrophages were allowed to adhere for 2 h. The non-adherent cells were washed off with PBS. In the adherent cell population ($\sim 2 \times 10^5$ cells/slide or $\sim 1 \times 10^5$ cells/well), 90% of the cells were macrophages as determined by their morphology following Giemsa staining. All the experiments were carried out in an incubator maintained at 25°C (± 0.1) with 5% CO₂. The cell viability was determined by trypan blue exclusion test. Approximately, 95% cells were viable prior to and after the experiments, indicating that the treatment did not affect the cell viability.

2.4. Preparation of yeast cell suspension

The heat-killed yeast cell suspension was made by warming commercial Baker's yeast (3 mg/ml PBS, pH 7.8) at 80°C for 15 min. The pellet was washed and resuspended in complete culture medium.

2.5. Phagocytic assay

For phagocytic assay, the macrophage monolayer was incubated with heat killed yeast cells for 90 min, washed with PBS, fixed in methanol, and stained with Giemsa. Without any predetermined sequence or scheme, ~ 200 macrophages/slide were observed. The percentage phagocytosis and the phagocytic index were calculated following the formulae: percentage phagocytosis—number of phagocytosing cells per 100 cells; phagocytic index—average number of yeast cells being phagocytosed by the phagocytic cell $\times$ percentage phagocytosis.

2.6. Superoxide assay

The intracellular superoxide anion was determined by the reduction of redox dye nitroterazolium blue chloride (NBT) as described by Sakai et al. (1996). In brief, macrophage monolayer was incubated in medium containing 1 mg/ml NBT and 1 $\mu\text{g}/\text{ml}$ PMA for 2 h. Cells were washed with PBS, fixed in methanol and the NBT reduction was stopped by adding 120 μl of 2 M KOH. The reduced product, formazan, in each well was dissolved in 140 μl of DMSO. The absorbance was measured at 620 nm by multiscan spectrophotometer.

2.7. Chemical sympathectomy

6-OHDA was dissolved in sterile 0.01% ascorbic acid/PBS and injected intraperitoneally (i.p.) as follows: (i) The first dose of 6-OHDA (10 mg/kg body wt.) was administered 7 days prior to sacrifice. (ii) On the next day (i.e. 6 days prior to sacrifice), a second dose of 15 mg/kg body wt. was injected. The last injection (60 mg/kg body wt.) was administered 1 day prior to sacrifice. Comparable amount (0.2 ml) of vehicle was injected in controls. The dose and injection schedule were finalized based on report in fishes (Dunel-Erb and Bailey, 1986).

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