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Stress hormones modulate lipopolysaccharide stimulation of head kidney interleukin-6 production in the catfish *Heteropneustes fossilis*: *In vivo* and *in vitro* studies

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ABSTRACT

Interleukin-6 (IL-6) is a pleiotropic cytokine secreted by immune tissues such as monocytes/macrophages and have pro-inflammatory/anti-inflammatory and neuroendocrine actions. In this study, we report the modulatory effects of stress hormones, the cortisol agonist dexamethasone and catecholamines on lipopolysaccharide (LPS) - induced stimulation of head kidney IL-6 in the catfish *Heteropneustes fossilis*. In the *in vivo* study, the intraperitoneal administration of LPS stimulated, and dexamethasone time-dependently inhibited IL-6 level. In the *in vitro* study, the incubation of macrophage cultures with LPS stimulated IL-6 level significantly in all incubation times. Dexamethasone did not alter the basal IL-6 level but inhibited time-dependently the LPS-induced stimulation. Likewise, catecholamines did not alter the basal level of IL-6. Both epinephrine and norepinephrine inhibited the LPS-induced stimulation of IL-6. Dopamine, on the other hand, was ineffective. The results indicate that IL-6 is a useful marker of head kidney macrophage activity for studying endocrine–immune interactions in the catfish.

1. Introduction

Fishes are exposed to a variety of stressors such as overcrowding, pathogens, parasites and toxicants compromising growth, immunity, disease resistance, reproduction and survival. The stress responses are mediated through the hypothalamus-pituitary-adrenocortical (HPA) axis and the sympatho-adrenomedullary (SAM) axis. The effector molecules are glucocorticoids, and catecholamines, respectively (Wendelaar Bonga, 1997; Padgett and Glaser, 2003). Among their targets, the stress hormones act on immune cells such as monocytes, macrophages, neutrophils and granulocytes through specific receptors, influencing their secretions (cytokines) (Weyts et al., 1998; Roy and Rai, 2008; Khansari et al. 2017a). The functional interactions between the neuroendocrine and immune systems are complex and essential to the maintenance of physiological homeostasis. Stress hormones are immunosuppressive and regulate the immune functions within the normal physiological range (Tort, 2011; Verburg-van Kemenade et al., 2011; Nardocci et al., 2014). The head kidney of teleosts is a unique structure containing both the adrenal and hemopoietic tissue, and this unique organization permits mutual functional interactions between hormones and immune molecules. In fishes, cortisol is the main glucocorticoid secreted by the interrenal cells (adrenocortical homolog) and epinephrine (EPI) and norepinephrine (NE) are the catecholamines secreted by the chromaffin tissue (adrenomedullary homolog).

Bacterial endotoxins like lipopolysaccharide (LPS) are responsible for pathogenicity of several diseases in fish (Fletcher and White, 1987). On one hand, LPS stimulates the stress (HPA) axis increasing cortisol secretion (Kumar, 2015; Nardocci et al., 2014) and on the other, as an immunostimulant, it stimulates immune cells to release proinflammatory cytokines like interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α (Bird et al., 2005; Call et al., 2000; Castellana et al., 2008; Castillo et al., 2009; Costa et al., 2011; Fast et al., 2007; Holland et al., 2002; Iliev et al., 2007; Khansari et al., 2017a,b; Nam et al., 2007; Nascimento et al., 2007). IL-1β, TNF-α and IL-6, which form a cytokine cascade, are responsible for both localized and systemic inflammatory responses (Breen, 2002). IL-6 is a pleiotropic cytokine modulating a variety of physiological events in vertebrates (pro- and anti-inflammatory role; endocrine, neural and hemopoietic actions; regulation of bone metabolism, steroidogenesis and blood pressure) (Castellana et al., 2008; Costa et al., 2011; Guzman et al., 2010; Varela et al., 2012). It is secreted by T cells, B cells, eosinophils, monocytes and endocrine tissues like adrenal, pituitary and gonads (Corripio-Miyar et al., 2012;

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Guzman et al., 2010). IL-6 is a phylogenetically ancient molecule conserved from fish to mammals and characterized in certain fish species (Bird et al., 2005; Iliev et al., 2007; Nam et al., 2007; Varela et al., 2012).

Studies on the role of stress hormones on IL-6 secretion by immune tissues in teleosts are limited and investigated at the mRNA transcript level (Castillo et al., 2009; Khansari et al., 2017a,b). Those studies also showed species variations. We have previously shown that LPS and stress hormones modulate phagocytosis and nitric oxide generation in head kidney macrophages (Kumar and Joy, 2018). In continuation of that study and in view of the versatile role of IL-6 in physiological functions, we investigated the effects of dexamethasone, a cortisol agonist and catecholamines on basal and LPS-induced stimulation of IL-6 in the head kidney and isolated macrophages at the protein level.

2. Materials and methods

2.1. Chemicals

EPI, NE, dopamine (DA), dexamethasone, percoll and lipopolysaccharide (LPS, *Escherichia coli*, 0127:B8, purified by phenol extraction, impurities: < 3% of protein) were purchased from Sigma-Aldrich, New Delhi, India. Human IL-6 ELISA assay kit was purchased from Orgenium Laboratories Business Unit, Finland. Fetal bovine serum (FBS), Leibovitz L-15 medium, tissue culture flasks for adherent cells (surface area 12.5 cm^2; total volume 25 mL) and syringe-driven filter (nylon hydrophilic membrane, pore size 0.45 µm, 30 mm diameter) were purchased from HiMedia Laboratories Pvt. Ltd, Mumbai, India. All other chemicals were of analytical grade and purchased from E. Merck (New Delhi, India) and Ranken (RFCL Limited, New Delhi, India).

2.2. Animals

Sexually mature *Heteropneustes fossilis* were collected from the Chaukaghat fish market in Varanasi during gonad resting (December-January) phase of the annual reproductive cycle. In the laboratory, the fish were acclimated for a week in cement tanks in the animal house. Female fish (30–35 g; 16.2–17.6 cm) were selected and acclimated in aquarium tanks under normal photoperiod (12 h light: 12 h dark) and ambient temperature (22.5 ± 2 °C; pH 7.2 ± 0.2). The fish were fed daily with boiled goat liver *ad libitum*.

The experiments were conducted as per the guidelines of the Animal Ethics Committee of Banaras Hindu University, Varanasi, India. All care was taken to prevent cruelty of any kind on animals.

2.3. In vivo experiments

2.3.1. Effect of LPS on IL-6 level

Lipopolysaccharide (LPS; 1 mg) was dissolved in PBS (pH 7.4). The stock solution was further diluted to make working solutions. To determine the effect of LPS on IL-6 level, a dose- and time- dependent study was done. Fish were divided into LPS (n = 30 fish) and control (n = 15 fish) groups. In the LPS group, 15 fish were injected intraperitoneally with LPS in a dose of 1.5 µg/g body weight (BW) and the remaining 15 fish were given LPS in a dose of 3 µg/g BW. In the control group, the fish were given the vehicle medium. From all the groups, 5 fish each were sacrificed after 4 h, 8 h and 12 h of the injections. The head kidneys were removed, weighed and stored at −80 °C for IL-6 assay (n = 5 samples for each time point).

2.3.2. Effect of dexamethasone on IL-6 level

Dexamethasone (1 mg) was dissolved in 10 µL of ethanol, and then diluted with PBS to make a 100 µL stock solution. Fish were divided into dexamethasone (n = 30 fish) and control (n = 15 fish) groups. Dexamethasone was injected intraperioneally in a dose of 1 µg/g BW to 15 fish and 10 µg/g BW to the remaining 15 fish. The control fish were given the vehicle. Five fish from each group were sacrificed after 4 h, 8 h and 12 h. The head kidneys were removed, weighed and stored at −80 °C until IL-6 was assayed (n = 5 samples for each time point).

2.3.3. Effect of both LPS and dexamethasone on IL-6 level

Eighty fish were used for this experiment and divided into 4 groups of 20 fish each. Group 1 was given both dexamethasone (10 µg/g BW) and LPS (3 µg/g BW). The remaining groups served as vehicle control, dexamethasone and LPS group positive controls. Five fish each from all the groups were sacrificed after 4 h, 8 h, 12 h and 24 h. The head kidneys were removed, weighed and stored at −80 °C until IL-6 was assayed (n = 5 samples for each time point).

2.4. In vitro experiments

Leibovitz (L-15) supplemented with 0.33% glucose was used as the incomplete L-15 medium. Complete Leibovitz medium was prepared by adding 5% FBS and 100 µg/mL streptomycin to the incomplete L-15 medium. Macrophages were isolated from head kidney, as described previously (Kumar and Joy, 2018).

2.4.1. Effect of LPS on IL-6 production by macrophages

The macrophage cultures ($1.5x10^6$ cells/mL) were treated with LPS (1 µg/mL) for 4 h, 8 h, 12 h or 16 h in a 5% CO_2 incubator at 20 °C. At the end of the incubations, the medium was collected and assayed for IL-6 (n = 5 samples for each time point).

2.4.2. Effects of dexamethasone alone or in combination with LPS on IL-6 production by macrophages

Macrophage cultures ($1.5x10^6$ cells/mL) were treated with dexamethasone alone (10 nM), LPS alone (1 µg/ml) or with both dexamethasone (10 nM) and LPS (1 µg/mL) for 4 h in a 5% CO_2 incubator at 20 °C. At the end of the incubations, the medium was collected and assayed for IL-6 (n = 5 samples in each group).

2.4.3. Effects of catecholamines alone or in combination with LPS on IL-6 production by macrophages

Stock solutions (1 mg/mL) of EPI, NE and DA were prepared in HPLC grade Milli-Q water in amber-colored vials. The stock solutions were diluted to make working solutions at the time of experiments. Macrophage cultures ($1.5x10^6$ cells/mL) were treated with EPI (10 nM), NE (10 nM), DA (10 nM) or LPS (1 µg/mL) for 4 h. Similarly, the macrophage cultures were incubated with LPS (1 µg/mL) + EPI (10 nM), LPS (1 µg/mL) + NE (10 nM) or LPS (1 µg/mL) + DA (10 nM) for 4 h. The incubations were carried out in a 5% CO_2 incubator at 20 °C. At the end of the incubations, the medium was collected and assayed for IL-6 (n = 5 samples in each group).

2.5. Interleukin-6 assay

The assay was carried out using the ELISA kit for IL-6 as per the manufacturer's protocol. The head kidney sample was dissolved in PBS buffer (pH 7.4), homogenized and centrifuged at 5000 rpm for 10 min at 4 °C. The supernatants were used for the IL-6 assay. Absorbance was taken at 450 nm using a microplate reader (EPOCH, Bio Tek Instruments Inc, Highland Park, USA). The concentration of IL-6 in samples was calculated using the standard curve. The standard curve was linear over the concentration range used (R^2 = 0.9914). The lowest sensitivity was 0.011 pg/mg tissue weight. The intra- and inter-assay variations were 5.82% and 7.9%, respectively.

2.6. Statistical analysis

The data were expressed as means ± SEM. The data were tested for normal distribution using the Shapiro-Wilk test and then analyzed by one way analysis of variance (ANOVA), followed by Newman-Keuls'

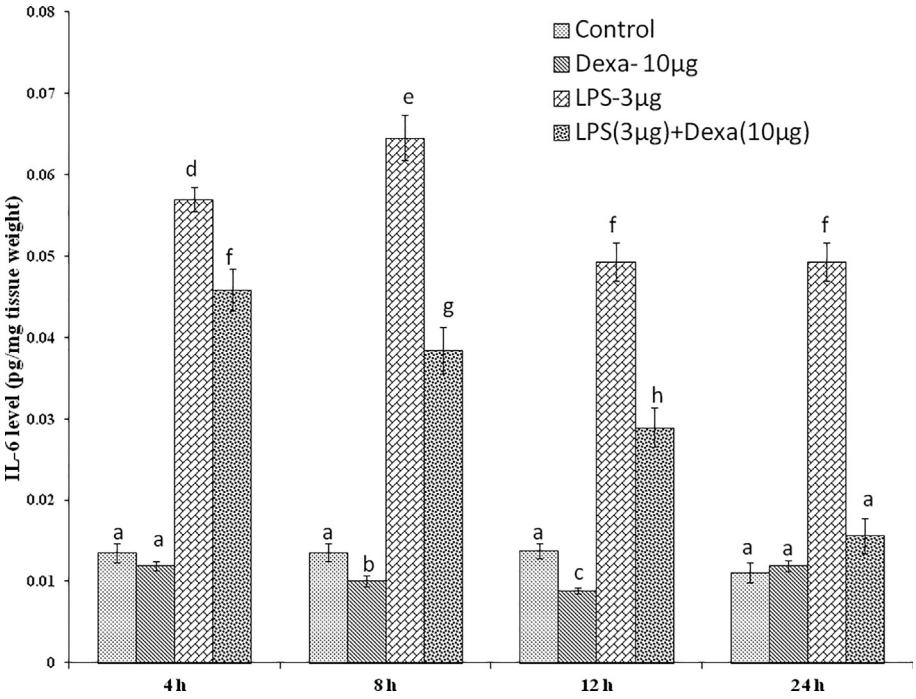

Fig. 1. *In vivo* effects of both LPS and dexamethasone on IL-6 level in the head kidney of the catfish *Heteropneustes fossilis.* Values are means ± SEM of 5 fish each. Data were analyzed by two way ANOVA ($p < 0.001$) and Newman-Keuls' test ($p < 0.05$). Groups bearing different letters are only significantly different.

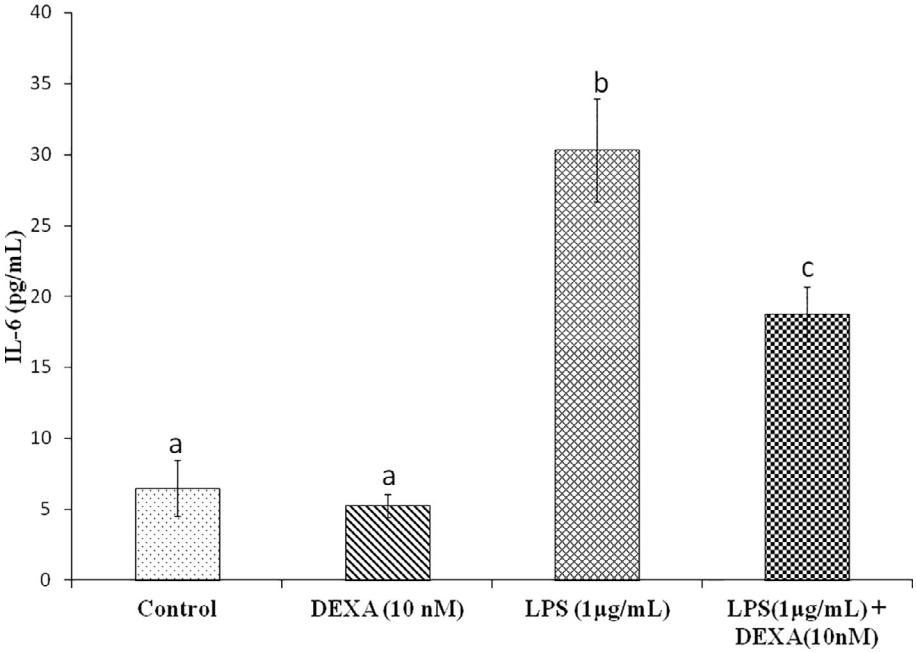

Fig. 2. *In vitro* effects of DEXA and LPS and their combination on IL-6 production head kidney macrophage cultures. Values are mean ± SEM of 5 replicates each. Data were analyzed by one way ANOVA ($p < 0.001$) and Newman-Keuls' test ($p < 0.05$). Groups bearing different letters are only significantly different.

test ($p < 0.05$) for multiple group comparisons. Data of LPS and dexamethasone dose– and time– dependent studies were analyzed by a two way ANOVA ($p < 0.001$). The analysis was performed using a SPSS version 16 for windows.

3. Results

3.1. In vivo experiments

3.1.1. Effects of LPS, dexamethasone and the combination on IL-6 levels

The administration of LPS increased the IL-6 level in a dose (1.5 µg/g and 3 µg/g) - and time-dependent manner at all the time duration (4, 8, 12 and 24 h) as compared with the control (Fig. 1, data for 3 µg/g dose only shown; $p < 0.001$; two way ANOVA, $F_{conc} = 345.65$; $F_{time} = 12.83$). The peak increase was noticed at 8 h ($p < 0.05$,

Newman-Keuls' test). The dexamethasone treatment (1 μg/g and 10 μg/g) decreased the IL-6 level in a dose- dependent manner as compared with the control (Fig. 1, data for 10 μg/g only shown; $p < 0.001$; two way ANOVA, $F_{conc} = 8.94$; $F_{time} = 1.92$). In the combination groups, the dexamethasone treatment resulted in inhibition of the LPS-induced increase in IL-6 level time-dependently. The inhibition at 4 h, 8 h and 12 h was partial with the levels significantly higher over the control value ($p < 0.05$, Newman-Keuls' test). But at 24 h, the IL-6 level was decreased to that of the basal level (Fig. 1).

3.2. *In vitro* experiments

3.2.1. *Effects of LPS, dexamethasone and the combination on IL-6 production*

The incubation of the macrophage cultures with LPS (1 μg/mL) for 4, 8, 12 and 16 h increased significantly the IL-6 level compared with the control group (data not shown). The peak increase was noticed at 4 h, and the levels decreased time-dependently but remained higher over the control values. The incubation of the macrophage cultures with dexamethasone (10 nM) did not alter the basal level of IL-6 compared to the control group (data not shown). However, the co-incubation of the macrophage cultures with LPS (1 μg/mL) and dexamethasone (10 nM) for 4 h inhibited the IL-6 level significantly compared to the LPS group (Fig. 2; $p < 0.05$, Newman-Keuls' test). However, the level remained significantly higher than the control group.

3.2.2. *Effects of catecholamines, LPS and the combination on IL-6 production*

The incubation of the macrophage cultures with EPI, NE or DA alone did not alter the IL-6 level at 4 h in comparison to the control group (Fig. 3). In the combination groups, only EPI and NE inhibited the LPS-induced increase in IL-6 level ($p < 0.05$, Newman-Keuls' test) and DA did not.

4. Discussion

In the present study, we measured IL-6 level in the head kidney (*in vivo*) and macrophage cultures (*in vitro*) to evaluate the effects of stress hormones. The LPS challenge stimulated the macrophages under both conditions. The presence of IL-6 in the head kidney may suggest a role of the cytokine in immune functions and also adrenal control, as has been reported in the adrenal glands of mammals (Call et al., 2000; Judd et al., 2000). Holland et al. (2002) reported the cytokine signaling of cortisol secretion in rainbow trout in response to injections of trout recombinant IL-1β or LPS. It is known that both IL-1β and TNF-α activates IL-6 expression (Duque and Descoteaux, 2014). Since LPS administration stimulated both cortisol (Kumar, 2015) and IL-6 (this study) levels in the head kidney, IL-6 may be a cytokine link in the activation of the HPA axis in fish.

In teleosts, the expression of *il-6* gene is up regulated by LPS and other immunostimulants like IL-1β, TNf-α, *Vibrio anguillarum* DNA, peptidoglycan, poly I:C, *Edwardsiella tarda* and phorbol dibutyrate (Bird et al., 2005; Call et al., 2000; Castellana et al., 2008; Costa et al., 2011; Iliev et al., 2007; Nam et al., 2007; Varela et al., 2012). The present *in vivo* and *in vitro* data corroborate the above observations at the protein level. While in the *in vivo* system the maximal response to LPS was obtained at 8 h in the *in vitro* model, the peak release was early at 4 h, suggesting a high sensitivity of the macrophages *in vitro* to LPS. Mackenzie et al. (2010) reported that peptidoglycans present as impurities in the commercial LPS (crude) is responsible for the cytokine production, pure LPS is nonstimulatory. Castillo et al. (2009) reported that cortisol (50 and 100 ng/mL) inhibited the expression of cytokine-related genes (*il-1β, tnf-α, il-6, tgf-β1*) in a dose-dependent manner in gilthead seabream (*Sparus aurata*) isolated head kidney cells. The LPS effect varied with the cytokine; stimulating (*il-1β*), inhibiting (*tgf-β1*) or ineffective (*tnf-α, il-6*). Therefore, all these cytokines should be evaluated in future studies to understand the cytokine responses in totality and the associated mechanisms.

In the catfish, the cortisol agonist dexamethasone inhibited the basal IL-6 level *in vivo* only and the LPS-induced increase of the IL-6 level, both *in vivo* and *in vitro*. The inhibition was time-dependent (4 h, 8 h and 12 h), attaining the basal level at 24 h. The inhibition of the basal IL-6 level by dexamethasone might be due to the suppression of ACTH secretion at the hypothalamus-pituitary level, as shown in the rainbow trout *Oncorhynchus mykiss* (Holland et al., 2002). In the catfish also, dexamethasone decreased the basal and LPS-induced rise in cortisol level (Kumar, 2015). Dexamethasone inhibited the innate response in rainbow trout (*Oncorhynchus mykiss*) experimentally infested with the microsporidian parasite *Loma salmonae* but did not reduce the adaptive immune response after the parasitic reinfection (Lovy et al., 2008). Khansari et al. (2017a,b) reported that cortisol and ACTH produced differential effects on cytokine-related gene expression in the head kidney primary cell cultures of the freshwater rainbow trout and the seawater gilthead seabream. These stress hormones decreased the expression of immune-related genes in the seabream but not in rainbow trout. Apparently, the environmental factors may interact with the HPA axis to mediate immune functions. Dexamethasone down regulated

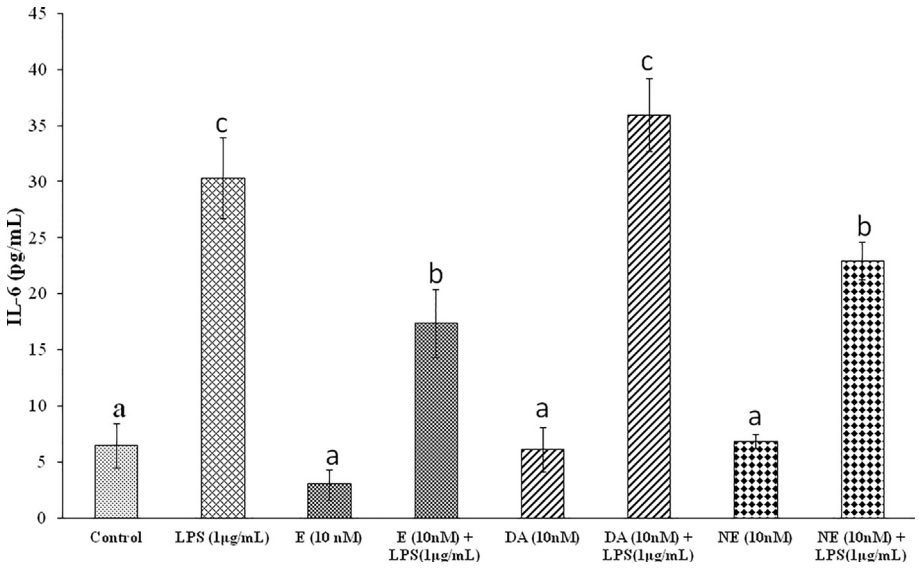

Fig. 3. *In vitro* effects of epinephrine (E), norepinephrine (NE) and dopamine (DA) on LPS-induced stimulation of IL-6 production in head kidney macrophage cultures. Values are mean ± SEM of 5 replicates each. Data were analyzed by one way ANOVA ($p < 0.001$) and Newman-Keuls' test ($p < 0.05$). Groups bearing different letters are significantly different.

hepatic and brain glucocorticoid receptors (GR) by lowering B_{max} and increasing K_d (receptor number and affinity) values in rainbow trout (*O. mykiss*) (Lee et al., 1992). GR with high affinity and low capacity binding for cortisol were reported in carp neutrophils (Weyts et al., 1998). In the catfish, the results of dexamethasone on IL-6 dynamics need to be compared with the endogenous hormone (cortisol) effect.

Adrenaline (EPI) responded differentially on the expression of cytokine genes in the rainbow trout and seabream (Castillo et al., 2009; Khansari et al., 2017a,b). EPI inhibited the expression of *il-1b* and *il-6* in the rainbow trout but increased the expression in the sea bream. In the seabream, the authors also reported that a short exposure to adrenaline (1 μM) inhibited *il-1β* expression but not *tnf-α* or *il-6*, and the longer exposure inhibited the expression of all these genes. These responses were reversed by the β-adrenergic receptor blocker propranolol, but not by the α-adrenergic receptor blocker phentolamine (Khansari et al. 2017a). In the catfish, catecholamines did not influence the basal level of IL-6 but differentially modulated the LPS-induced stimulation. Both EPI and NE inhibited partially the LPS-induced stimulation. On the other hand, DA did not alter the LPS- increased IL-6 level significantly. DA has been shown to stimulate basal IL-1β, IL-6 and IL-8 release in rat adrenal glomerulosa primary cell cultures and human keratinocytes (Ritchie et al., 1996; Parrado et al., 2012).

5. Conclusion

Stress hormones elicit an inhibitory effect on LPS-induced stimulation of IL-6 production by head kidney/cultured macrophages, suggesting their modulatory actions on macrophage activity. Catecholamines exert differential roles: EPI and NE are inhibitory, while the DA effect was insignificant. Future studies involving the direct action of the endogenous hormone cortisol on the macrophage cytokines and receptor characterization will help to understand the cytokine responses in totality and associated receptor mechanisms.

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