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The Proliferative and Apoptotic Properties of Nile Tilapia (*Oreochromis niloticus*) sIg$^+$ Lymphocytes by Cortisol Treatment

Kwan-Ha Park and Sanghoon Choi*

Department of Aquatic Life Medicine, Kunsan National University, Gunsan 573-400, Korea

ABSTRACT

The effects of cortisol on proliferation and apoptosis of tilapia surface immunoglobulin positive (sIg$^+$) lymphocytes isolated from different tissues were investigated. sIg$^+$ lymphocytes from the tilapia head kidney (HK) and spleen showed a higher proliferation and lower intracellular calcium (Ca^{2+}_i) level to Ig-crosslinking compared with peripheral blood sIg$^+$ lymphocytes. Peripheral blood sIg$^+$ lymphocytes stimulated with lipopolysaccharide (LPS) showed high levels of apoptosis in the presence of cortisol. HK and to a lesser extent spleen sIg$^+$ lymphocytes, although less sensitive than their equivalent in peripheral blood, showed cortisol-induced apoptosis irrespective of LPS stimulation of control levels. Compared to plasma values measured during stress conditions, proliferation regardless of LPS stimulation was apparently suppressed by cortisol that is effective in inducing a significant increase in apoptosis in all three different cell populations of sIg$^+$ cells, suggesting the immunoregulatory effect of cortisol in both LPS stimulated and non-stimulated conditions. Different sensitivity of sIg$^+$ cells to the cortisol, in regard to developmental stage and activity, could be related in inhibiting excessive and continuing depletion of sIg$^+$ lymphocytes.

(**Key words :** Nile tilapia, sIg$^+$ lymphocytes, Cortisol, Apoptosis, Proliferation)

INTRODUCTION

Corticosteroids consist of the glucocorticoids (GCs) and the mineralocorticoids that are synthesized in the adrenal cortex in mammals and in the interrenal tissue of the HK in teleost (Bury et al., 2003). In teleosts the main active corticosteroid is believed to be cortisol (Aluru and Vijayan, 2009; Castro et al., 2011) and is regarded as the most important transducer of the stress response in fish. It induces apoptotic and necrotic cell death of leucocytes in a number of species (Laing et al., 2001; Yada and Nakanishi, 2002; Saha et al., 2003) and decreases the phagocytic response, mitogenesis, antibody production, lymphocyte numbers and resistance to pathogens (Yada and Nakanishi, 2002; Saha et al., 2003). To develop disease control procedures in aquaculture, it is highly crucial to reveal the mechanism of the neuroendocrine-immune system interactions in fish. Stressor-induced immunomodulation has mainly been attributed to cortisol (Ellis, 1981; Barton et al., 1991), the major corticosteroid in many fish, which is produced by cells in the interrenal tissue. Cortisol secretion is under endocrine control from the pituitary and the main mediators are ACTH and αMSH, which are enhanced during acute and chronic stress (Wendelaar Bonga, 1997).

Cortisol treatment to fish resulted in reduction of: (1) leukocyte proliferation (Ellsaesser and Clem, 1987; Le Morvan-Rocher et al., 1995; Espelid et al., 1996); (2) numbers of antibody producing cells (Carlson et al., 1993; Mazur and Iwama, 1993); (3) levels of virus-neutralizing antibodies (Wechsler et al., 1986) and (4) circulating numbers of lymphocytes (Ellsaesser & Clem, 1987; Espelid et al., 1996). The mechanism of cortisol-induced immunosuppression has been the subject of several studies. The literature concerning the impact of stressors and corticosteroids on activity of phagocytic cells is not consistent, probably due to the difference in species, stress-protocols and assay systems (Weyts et al., 1999). *In vitro* studies have revealed that mainly B-lymphocytes appear to be directly affected by cortisol, manifested by reduced levels of proliferation (Grimm, 1985; Tripp et al., 1987; Espelid et al., 1996) and reduced antibody production (Tripp et al., 1987). Moreover, it has been shown that carp peripheral lymphocytes and HK neutrophilic granulocytes possess high affinity receptors for cortisol and that at least part of the impact of cortisol is

* Corresponding author : Sanghoon Choi; Department of Aquatic Life Medicine, Kunsan National University, Gunsan 573-400, Korea, Tel: 063-469-1886, Fax: 063-63-9493, E-mail: shchoi@kunsan.ac.kr

mediated through these receptors and affects apoptosis in these cells (Weyts et al., 1998a). These effects of cortisol on cell viability are cell type specific and may be dependent on the differentiation and activation state of the leukocytes. Stimulated B-lymphocytes are especially sensitive and easily become apoptotic, whereas thrombocytes and cells of the T-lymphocyte fraction are insensitive to cortisol (Weyts et al., 1997, 1998b). In contrast, apoptosis of HK neutrophilic granulocytes was inhibited when cultured in the presence of cortisol (Weyts et al., 1998c). Clearly, as neutrophils, together with macrophages form a first line of defense against invasion by micro-organisms, mobilization of these cells in conditions of stress may be important for survival.

The high sensitivity of peripheral sIg^+ lymphocytes, especially in the activated state, evoked our interest to study sIg^+-cell populations from the tilapia haematopoietic HK (with high numbers of developing lymphocytes) and spleen as a secondary lymphoid organ, as well as from the peripheral circulation. Lymphocyte populations were characterized for surface immunoglobulin (sIg) expression, basal and lipopolysaccharide (LPS) stimulated proliferation and apoptosis, and effect of Ig-crosslinking on increases of intracellular calcium concentration. Subsequently the effect of cortisol was analyzed against non-stimulated and LPS-stimulated proliferation and apoptosis.

MATERIALS AND METHODS

1. Fish

Adult tilapia, *Oreochromis niloticus,* of 30~40 g were obtained from the fish farm at Kunsan National University, Kunsan, Korea and maintained in FW recirculation tanks supplied with a filter and aeration system at 22~23℃. Pellet food was fed at a daily rate of 0.5% of their body weight. At the end of the experiment, tilapia were immediately anesthetized and sacrificed with 0.2 g/l tricaine methane sulphonate.

2. Leukocytes isolation

To harvest tilapia lymphocytes, tilapia HK and spleen were dissected out by a ventral incision, cut into small fragments and transferred to 5 ml Hank's balanced salt solution (HBSS), respectively. Cell suspensions from HK and spleen were obtained by teasing the tissues with 2 slide glasses in HBSS in a Petri dish (Coring, USA). After sedimentation of tissue debris at 4℃ for 1 min, the supernatants were removed. HK and spleen cell suspensions were layered over a 34~51% Percoll gradient and centrifuged at 1000 × g for 40 min at 14℃. After centrifugation, the bands of leukocytes above the 34~51% interfaces were collected with a Pasteur pipette and washed twice at 120 × g for 8 min in HBSS. The concentration of viable cells was determined by trypan blue exclusion. All cell suspensions were plated in 24 well culture plates at a density of 10^7 cells/well and left to adhere for 1 h at 26℃ and 5% CO_2, to remove neutrophilic granulocytes and monocytes/ macrophages. Non-adherent cells were subsequently harvested by carefully pipetting off and suspended at a density of 10^7 cells/ml.

3. Culture of cells

Cells were seeded in 96-well plates (10^6 cells/well) and cultured overnight in Roswell Park Memorial Institute medium (RPMI) containing 10^6 IU/ml penicillin-G (Sigma, USA), 50 mg/l streptomycin sulphate (Serva, Germany) and 2.0 mmol/l L-glutamine. Cells received no stimulus, or were stimulated with lipopolysaccharide (LPS 100 ng/ml; *E. coli*: B5 LPS, Difco, Detroit, MI, USA) for 4 h at 27℃ in 5% CO_2, followed by the addition of 0.5% pooled tilapia serum (PTS, pooled serum from 20 adult tilapia, containing 45 ng cortisol/ml, as determined by radioimmunoassay). Cortisol (36 ng/ml or 10^{-7} M) was added and cultures were maintained for 24 h and 48 h at 27℃ in 5% CO_2. This cortisol concentration corresponds to half maximal free plasma cortisol concentrations in mildly stressed fish and induces substantial apoptosis in active peripheral blood lymphocytes (PBL) *in vitro* (Weyts et al., 1997).

4. Measurement of sIg^+ leukocytes

Leukocytes (1.25×10^6/ml) were incubated for 30 min at 4℃ with a monoclonal antibody (mAb) against flounder IgH chain, FIM 511 which was previously manufactured in our laboratory and showed the cross-reactivity with tilapia IgH chain (Jang et al., 2004). Cells were washed and centrifuged for 7 min at 680 × g at 4℃. They were resuspended in RPMI followed by incubation with fluorescein-isothiocyanate (FITC)-conjugated or rhodamine-conjugated rabbit-anti-mouse IgG (RAM-Ig) antibody (1:100; Dako A/S, Glostrup, Denmark)

for 20 min at 0℃. After washing, 10^4 cells were analyzed using a FACStar (Becton Dickinson, Mountain View, CA, USA) tuned at 488 nm using the DataMATE software (Applied cytometry systems, USA). Within the lymphocyte gate (Kouman-van Diepen et al., 1994), the percentage of cells stained with the antibodies was determined.

5. Intracellular calcium levels

Measurement of changes in intracellular calcium was performed as established earlier (Verburg-van kemenade et al., 1998). Lymphocytes were loaded with fluorescent Ca indicator at a cell density of 10^7/ml, at room temperature in the dark. Fluo3-AM, 4 μmol/l (Sigma, USA) was added from a 1 mM stock solution in dry dimethylsulfoxide (DMS). To improve the uptake-efficiency 6 μl/ml of Pluronic F-127 (Sigma, USA, 3% w/v in RPMI) was added. After 40 min, the loading solution was diluted 1:10 with RPMI, and after incubation for another 10 min, the cells were collected by centrifugation for 7 min at $700 \times g$. The pellet was resuspended to obtain 1.25×10^6 cells/ml in RPMI. Cells were then incubated at 26℃ before and during $Ca^{2+}{}_i$ analysis.

Fluo-3 emission fluorescence in the cells was recorded with the flow cytometer at 530 ± 30 nm. Baseline fluorescence was established at 5 min intervals (experimental samples were measured in paralleled at 30s intervals). After cross-linking of sIg by addition of FIM 511 and RAM-Ig (Dakopat, Denmark) the fluorescence intensity was reassessed within a 10s time span and every 5 min thereafter.

6. Proliferation assay

The cultured cells were adjusted to 3×10^6 cells/ml in 10% FBS-DMEM, and then 3×10^5 cells/100 μl were dispensed into wells of 96 well plate. The cells were incubated for a further 72 h prior to the addition of 0.5 μCi[^{3}H] thymidine into the wells containing proliferating cells. After 12 h, the cells were harvested using an automash 2000 cell harvester (Dynatech, USA) trapping their DNA onto glass filtermates. Dried filter circles were placed in plastic scintillation counter vials (Packard, USA). All cpm values were subtracted by the background cpm values of non-stimulated cultures. All samples were prepared in triplicate.

7. Apoptosis assay

Following FIM 511 labeling as described above, cells were washed in RPMI supplemented with 1% BSA and 0.01% sodium azide. They were labeled with annexin V, conjugated to FITC (Boehringer, Mannheim, Germany), as described by the manufacturer. Annexin V has been shown to detect apoptosis in carp lymphocytes (Weyts et al., 1998b). Green and red fluorescence intensities of cells within the lymphocyte gate were measured in the FACScan. In a parallel sample, propidium iodide (PI) exclusion was used to distinguish necrotic cells, which also expose phosphatidyl-serine from apoptotic cells. Measurements per fish were performed in duplicate.

8. Statistics

The statistical significance of differences between groups was calculated by applying Student's 2-tailed t-test.

RESULTS AND DISCUSSION

Weyts et al. (1998a) reported that cortisol is involved in immune regulatory mechanism of carp. Furthermore, sIg^+ cells within the PBL are especially sensitive to cortisol, which induced strong dose-dependent decrease in the level of proliferation and a massive onset of apoptosis. However, these cells only became sensitive following stimulation, confirming previous results (Weyts et al. 1998b). Under stressful state, implying high levels of endogenous cortisol, one would argue that the generation of an efficient humoral response would thus be severely affected. In the absence of immune stimuli, e.g. invading microorganisms, the circulating cell population would be relatively protected due to lower sensitivity to the corticosteroid. This is indicative of a function of cortisol in removal of activated lymphocytes following an immune response. These lymphocytes may be potentially harmful with respect to a greater chance of unwanted autoimmune reactions. Conditions of stress might cause this process to take place too rapidly. The high relative cortisol-induced reduction of proliferation in the non-stimulated PBL population seems to contradict this fact. This, however, may be explained by the fact that proliferation capacity of non-stimulated PBL is extremely low in absolute amount, and thus is most probably ascribed to a very small percentage of activated and thus cortisol-sensitive

cells within this population.

The percentages of sIg^+ cells in the cell suspensions of non-adherent cells from HK, spleen and blood amounted to 32% (±4.2), 27% (±2.3) and 49% (±3.7), respectively (data not shown). Basal *in vitro* proliferation capacity and sensitivity to LPS stimulation is given in Fig. 1. Highest basal proliferation was found in cells of HK and PBL, whereas spleen lymphocytes showed low proliferation. After 1 day of LPS stimulation, proliferation was increased significantly in HK and PBL. The significantly enhanced proliferation was again found in HK and PBL after 2 days and HK only after 3 days of cell culture whereas spleen lymphocytes showed low levels of proliferation (Fig. 1a). HK leukocytes proliferation was significantly higher after 2 days of cell culture in the presence of LPS. Although HK leukocytes after 3 days culture with LPS failed to show higher proliferation pattern than those after 2 days culture, significantly elevated proliferation was found compared to the control in the absence of LPS. The result suggests that LPS is more sensitive in evoking proliferation of cell in kidney than in PBL and spleen.

As the effect of sIg-crosslinking on calcium induction in cytoplasm of HK, PBL and spleen was investigated, the sIg^+ cells in HK leukocyte suspensions reacted to sIg-crosslinking with the highest elevation of intracellular calcium levels as compared to spleen cells and PBL (Fig. 1b). Basal apoptosis values directly after cell isolation were lowest in PBL (10.3% ± 2.4) (data not shown). After 1, 2 or 3 days of cell culture, however, apoptosis levels highly increased and were highest in PBL. HK B cells showed the lowest level of apoptosis regardless of culture periods. After LPS treatment for 1 day, levels of apoptosis significantly decreased in all cell populations, whereas spleen failed to show a reduction of apoptosis even after 2 or 3 days of cell culture with LPS (Fig. 1c), indicating that spleen cells were not reactive to LPS in inducing cell proliferation.

Cortisol decreased the *in vitro* proliferation capacity of all cell populations (Fig. 2). After 1, 2 and 3 days of culture with cortisol, HK showed higher percentages of relative proliferation to control than other cell population suggesting HK cells were more resistant to cortisol than other cell populations. Spleen cells were more affected by cortisol, showing a 37%, 16% and 15% of relative percentage of proliferation after 1, 2 and 3 days of culture time, respectively. Peripheral blood leukocytes, which had a very low basic proliferation capacity *in vitro*, showed a lowest relative percentage of proliferation after 1, 2 and 3 days of culture. Combined cortisol and LPS treatment increased absolute proliferation *in vitro*, but resulted in a relatively higher cortisol induced reduction of proliferation as compared to non-stimulated cells. The impact of the stressors on immune competence cannot solely be explained by effects of cortisol on circulating lymphocytes; it will of course also depend on the effect of cortisol on the populations of developing sIg^+ cells in the haematopoietic organs. With respect to proliferation capacity it may be concluded that in HK high cortisol levels of relatively short duration (<1 day) may have limited impact. However, longer cortisol treatment

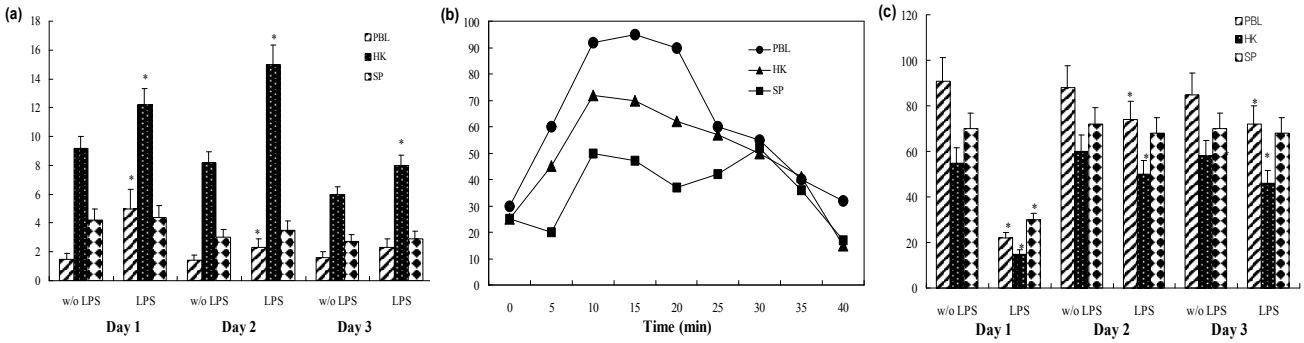

Fig. 1. (a) ^3H Thymidine incorporation assay for non-adherent leukocytes from PBL, HK and spleen (SP) with or without LPS treatment after 1, 2 and 3 days of culture. Bars represent the means of 4 fish ± S.E. (b) Intracellular Ca^{2+} levels in non-adherent leukocytes of PBL, HK and spleen after Ig-crosslinking with FIM511. (c) Percentage of apoptotic cells in non-adherent leukocyte populations from PBL, HK and spleen with or without LPS treatment measured at 0, 1 and 2 days in culture. Bars represent the means of 8 fish ±S.E. Statistical differences ($p<0.01$ and $p<0.05$) between groups are indicated by asterisks over the bar.

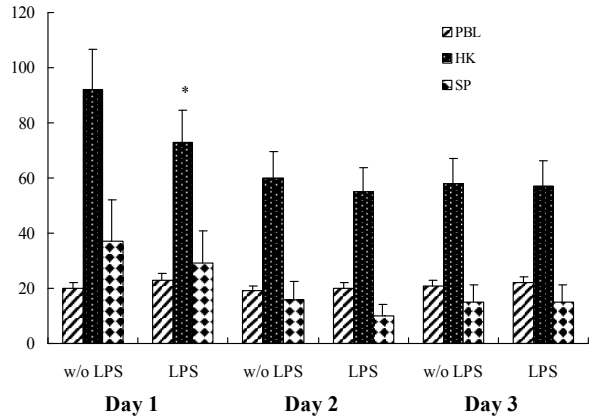

Fig. 2. Relative decrease of *in vitro* proliferation (^{3}H-thymidine incorporation) after 1, 2 and 3 days of culture in the presence of cortisol in non-adherent leukocytes from peripheral blood, HK and spleen. Bars represent the means of three fish ± S.E. Cortisol inhibited proliferation in all organs ($p<0.01$) and all organs differed in their sensitivity to cortisol ($p<0.01$) at different incubation periods regardless of LPS treatment. Statistical differences ($p<0.01$ and $p<0.05$) between groups are indicated by asterisks over the bar.

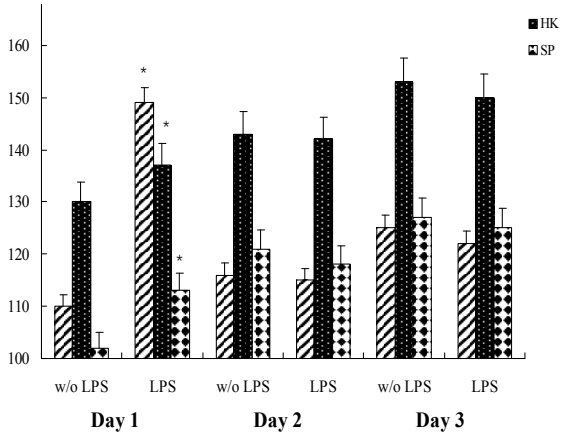

Fig. 3. Relative increase of apoptotic (AnnexinV positive) cells in non-adherent leukocyte populations from peripheral blood, HK and spleen after 1, 2 and 3 days *in vitro* culture in the presence of cortisol. Control means the culture of non-stimulated cells. LPS represents the cell cultures stimulated with LPS. Bars represent the means of 8 fish ± S.E. LPS treated sample are significantly different from the control at $p<0.01$ only after 1 day of culture. Statistical differences ($p<0.01$ and $p<0.05$) between groups are indicated by asterisks over the bar.

may be more harmful, as a 2 and 3 days treatment resulted in reduction of proliferation in both stimulated and non-stimulated conditions. On the other hand, compared to the results found with both spleen cells and PBL, HK sIg^+ cells are likely to be most protected.

After 1 day of culture, stimulation with LPS appeared to be very sensitive to cortisol treatment in PBL, whereas both HK and spleen leukocytes showed an intermediately enhanced level of apoptosis (Fig. 3). Regardless of LPS treatment, however, no significant differences of apoptosis were observed on the post 2 and 3 days of culture in all different cell populations. In regard to apoptosis the results show that in contrast to non-stimulated PBL, HK B cells and to a lesser extent spleen B cells are sensitive to cortisol-induced apoptosis. This may be indicative of a role of cortisol in B-cell selection. Further stimulation with LPS *in vitro* hardly induced any extra effects. In mammals, immature T and B cells are easily induced into apoptosis by glucocorticosteroids, consistent with the role of the steroids in the selection process (Ashwell et al., 1996; Lenardo, 1997). For fish this issue has not yet been investigated.

Understanding the mechanism of stressor-induced immunomodulation in teleosts is important to improve culture conditions in regard to negative effects of crowding, handling and transport. It is very hard to figure out which stressor-induced effects are cortisol mediated. Moreover, considering the findings that physiologically low-stress concentrations of cortisol are effective in inducing increased apoptosis and inhibited proliferation, it may be concluded that cortisol-induced immunomodulation is an integral part of immune cell development and immunoregulation in fish, independent of stressors. In this respect it may be relevant that the HK in fish harbors both the interrenal steroidogenic cells and the haematopoietic cells, possibly enabling paracrine interactions between both cell types. For tilapia it is concluded that the sensitivity of B cells to cortisol is dependent on the state of activation and/or development. This differential regulation in different immune organ may be important for physiological regulation of the total immune response in stress as well as non-stress circumstances.

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