INTRODUCTION

Immunosuppressive viral diseases threaten the poultry industry by increasing susceptibility to secondary infections and suboptimal response to vaccinations (Berg, 2000; Islam et al., 2001). Immunosuppressed flocks result in secondary infections, suboptimal response to vaccinations, and growth retardation, causing a great deal of economic loss to the poultry industry. Therefore, nutrition strategies may be desirable to alleviate immunosuppression in broiler chickens.

A possible application of immunomodulators, such as conjugated linoleic acids (CLA) of relevance immunosuppression were investigated. To test the hypothesis that CLA ameliorate immunosuppression, we developed the immunosuppressive model of peripheral blood T lymphocytes in broiler chickens induced by cyclosporin A. Peripheral blood T lymphocytes of broiler chickens were cultured with media containing various concentrations (25, 50, 100, and 200 µmol/L) of c9, t11-CLA and t10, c12-CLA to investigate the effects of CLA isomers on peripheral blood T lymphocyte proliferation, interleukin-2, the activity of phospholipase C, and protein kinase C production. Results suggested that CLA alleviated the immunosuppression of T lymphocytes in broiler chickens exposed to cyclosporin A through increasing of peripheral blood T lymphocyte proliferation and interleukin-2. The 2 CLA isomers enhanced T lymphocyte proliferation at low concentration and inhibited T lymphocyte proliferation at high concentration. In addition, the effect of c9, t11-CLA was better than that of t10, c12-CLA. At the cellular level, the effects of CLA on the alleviation of immunosuppression in T lymphocytes are mainly attributable to increasing the signaling molecules, such as phospholipase C and protein kinase C.

Key words: conjugated linoleic acid, immunosuppression, signaling molecule, chicken

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created a model of CsA in T lymphocytes and determined the effects of dietary CLA on the lymphocyte proliferation, IL-2, the activity of phospholipase C (PLC), and protein kinase C (PKC) production.

MATERIALS AND METHODS

Dietary Treatments and Bird Management

The animal management protocol for this research was approved by the China Agricultural University Animal Care and Use Committee. Eighty 1-d-old male Arbor Acre broiler chickens were randomly allocated into 1 group with 8 replicates, 10 chicks per replicate (cage). A corn-soybean meal diet was used. Birds were fed for 3 wk. Chicks had free access to feed and water and were housed in wire cages and maintained on a 24-h constant-light program. Temperature in the chicken house was set at 35°C for the first 3 d and was reduced by 3°C each consecutive week until it reached 24°C. Compositions of the diets and nutrient levels for starters (d 1–21) are presented in Table 1. The diets were formulated to meet or exceed the NRC requirements.

Sample Collection

On d 21, one bird was randomly selected from each replicate, and heparinized blood samples were collected from the wing vein by venipuncture to measure the proliferation, IL-2 production, the activity of PLC, and PKC production of peripheral blood T lymphocytes immediately.

Preparation of Fatty Acids

Both isomers of CLA and linoleic acid (LA; ≥98% pure; Matreya Inc., Pleasant Gap, PA) were complexed to fatty acids-free at a 3:1 molar ratio using 1 mmol/L of BSA stocks as previously described (Ringseirs et al., 2008).

T Lymphocyte Isolation, Culture, and Treatment

Heparinized blood samples were collected from the wing vein. Lymphocytes were isolated from peripheral blood using lymphocyte density-gradient centrifugation medium (density = 1.077; HaoYang Biological Manufacture Co. Ltd., Tianjin, China). Lymphocytes were suspended in RPMI 1640 medium (Gibco, Grand Island, NY) containing 5% fetal calf serum and 25 mM HEPES (pH 7.0), and then applied to a nylon fiber column (Dainippon Pharmaceutical, Osaka, Japan) for purifying T lymphocytes according to the manufacturer’s instructions. After that, T lymphocytes were washed 3 times in RPMI 1640 medium before being resuspended in RPMI medium supplemented with 5% fetal calf serum. Cell concentration was adjusted to 1 × 10^7 cells/mL of culture medium. One hundred microliters of cell suspension in a 96-well microtiter plate (Costar 3599, Corning Inc., Corning, NY) was incubated with lymphocyte mitogen concanavalin A (Con A; 10 µg/mL) in combination with CsA (20 ng/mL; Sigma Chemical Co., St. Louis, MO) for 2 h at 37°C with 5% CO2 in an incubator. After incubation with CsA, cells were incubated with CLA isomers or LA for another 70 h. Experiments were carried out in triplicate and repeated at least twice.

The lymphocyte proliferation assay was performed as described previously (Lambrecht et al., 2004; Zhang et al., 2005; Rauw et al., 2007).

IL-2 Production of Peripheral Blood T Lymphocyte

A 950-µL cell suspension was placed into a 24-well plate and incubated with lymphocyte mitogen Con A (10 µg/mL) in combination with CsA (20 ng/mL) for 2 h at 37°C with 5% CO2 in an incubator. After incubation with CsA, cells were incubated with CLA isomers or LA for another 46 h. Experiments were carried out in triplicate and repeated at least twice. Then, the supernatant was harvested and stored at −30°C until analyzed. Interleukin-2 production in lymphocyte culture supernatants was determined by IL-2 RIA kit (RapidBio Inc. 23830, Calabaras, CA), according to the manufacturer’s instructions.

The Activity of PLC

The cells were collected and suspended in 1 mL of homogenate solution (pH 7.2) and homogenized in ice. After centrifugation at 750–800 × g at 4°C for 10 min, the supernatant was centrifuged again at 10,000 × g at 4°C for 1 h. The pellets were resuspended and treated by sonication several times for 2 min at 4°C and used as membranous PLC enzymes. Enzyme activity was ex-

Table 1. Ingredients and compositions of dietary treatments on as-fed basis (%)

| Ingredient       | %  | Nutrition level | %  |
|------------------|----|-----------------|----|
| Maize            | 55.32 | ME (Mcal/kg) | 2.95 |
| Soybean meal     | 37.26 | CP             | 21.00 |
| Soybean oil      | 3.33  | Calcium        | 1.00  |
| Dicalcium phosphate | 1.98  | Available phosphorus | 0.45 |
| Limestone        | 1.18  | Methionine     | 0.50  |
| Sodium chloride  | 0.35  | Lysine         | 1.15  |
| Mineral premix1  | 0.20  |                |      |
| Choline chloride | 0.16  |                |      |
| Methionine       | 0.18  |                |      |
| Lysine-HCl       | 0.051 |                |      |
| Vitamin premix2  | 0.03  |                |      |
| Antioxidants     | 0.02  |                |      |

1Provided per kilogram of diet: Cu, 8 mg; Mn, 75 mg; Fe, 80 mg; Mn, 100 mg; Se, 0.15 mg; I, 0.35 mg.
2Provided per kilogram of diet: vitamin A, 12,500 IU; vitamin D3, 2,500 IU; vitamin K3, 2.65 mg; thiamine, 2 mg; riboflavin, 6 mg; vitamin B12, 0.025 mg; vitamin E, 30 IU; biotin, 0.0325 mg; nicotinic acid, 1.25 mg; pantothenic acid, 12 mg; niacin, 50 mg.
pressed as nanomoles per minute per milligram of protein (nmol/min per mg). The PLC assay was performed as described previously (Wu et al., 1997).

**PKC Assay**

The cells were placed into a 24-well plate and incubated for 48 h with 10 µg/mL of Con A at 37°C with 5% CO₂ in an incubator. The supernatant was harvested and stored at −30°C until analyzed. The PKC production in T-lymphocyte culture supernatants was determined by a PKC kit (Promega Co., Madison, WI), according to the manufacturer’s instructions.

**Statistical Analysis**

Data were reported as means and standard deviations and analyzed by one-way ANOVA of SPSS 10.0 (SPSS Inc., Chicago, IL). The significance of differences among different groups was evaluated by a least significant difference post hoc multiple comparisons test. A level of \( P < 0.05 \) was set as the criterion for statistical significance.

**RESULTS**

**CsA-Induced Immunosuppressive Model in Peripheral Blood T Lymphocytes**

The CsA-induced immunosuppressive effect of T lymphocyte proliferation in response to Con A mitogen is given in Figure 1. With the concentration gradient increase of CsA (0, 10, 20, 40, 60, 80, 100, 200, 300 ng/mL), T lymphocyte proliferation was significantly decreased (\( P < 0.05 \)). Thus, in the following study, CsA at the concentration of 20 ng/mL was used to set up the model of immunosuppression.

**Peripheral Blood T Lymphocyte Proliferation**

The results showed that CsA significantly inhibited (\( P < 0.05 \)) the peripheral blood T lymphocyte proliferation, and c9, t11-CLA and t10, c12-CLA alleviated the CsA-induced immunosuppression through increasing peripheral blood T lymphocyte proliferation in response to Con A mitogen (Figure 2). To some extent, under the CsA-induced immunosuppressive status, the 2 CLA isomers enhanced T lymphocyte proliferation at low concentration and inhibited lymphocyte proliferation at high concentration. In addition, the effect of c9, t11-CLA was better than that of t10, c12-CLA. The suitable concentrations of c9, t11-CLA were 25 and 50 µmol/L. Therefore, in the following studies, we only analyzed the effects of 25 µmol/L of c9, t11-CLA and t10, c12-CLA on the determinations of IL-2, PLC, and PKC in peripheral blood T lymphocyte.

**IL-2 Production of Peripheral Blood T Lymphocyte**

Compared with the control, CsA tended to inhibit IL-2 production of peripheral blood T lymphocyte (\( P = 0.113 \)). Both CLA isomers at the concentration of 25 µmol/L tended to increase (\( P = 0.081 \)) IL-2 production of T lymphocyte-induced immunosuppression by CsA (Figure 3). Thus, the results could indicate that c9, t11-CLA and t10, c12-CLA alleviated the CsA-induced immunosuppression.

**The Activity of PLC in Peripheral Blood T Lymphocyte**

Compared with the control, CsA had no effect on the activity of PLC of peripheral blood T lymphocyte (Fig-
The c9, t11-CLA isomer significantly increased \((P < 0.05)\) the \(Ca^{2+}\)-dependent and -independent PLC activities in the particulate and cytosolic fraction of peripheral blood T lymphocyte treated with CsA. The t10, c12-CLA tended to increase the activity of \(Ca^{2+}\)-independent PLC in the particulate and significantly increased \((P < 0.05)\) \(Ca^{2+}\)-dependent and -independent PLC activities in the cytosolic fraction of peripheral blood T lymphocyte treated with CsA. And the c9, t11-CLA isomer had better effects on the activities of PLC of peripheral blood T lymphocyte treated with CsA than that of t10, c12-CLA.

The Production of PKC in Peripheral Blood T Lymphocyte

The CsA significantly decreased \((P < 0.05)\) the production of PKC of peripheral blood T lymphocyte (Figure 5). Compared with the t10, c12-CLA isomer, c9, t11-CLA isomer significantly increased \((P < 0.05)\) the production of PKC of peripheral blood T lymphocyte treated with CsA.

DISCUSSION

Many studies have shown a critical role of dietary CLA in the modulation of immune function in animals such as rat, pigs, and chickens (Sugano et al., 1998; Yamasaki et al., 2000; O’Shea et al., 2004; Zhang et al., 2005). The immune function of CLA was not only in the normal physiological status but also the immunosuppressive status (Bassaganya-Riera et al., 2003). In this study, to examine whether the supplementation of CLA attenuated the immunosuppression at the cellular level, we first used a model for inducing immunosuppression of peripheral blood T lymphocyte in chickens by culturing with media containing CsA. The CsA was reported to primarily suppress T lymphocyte proliferation. Here, we found that CsA significantly decreased the peripheral blood T lymphocyte proliferation, which was in line with the previous studies (Raj and Jones, 1997; Khehra and Jones, 1999; Isobe et al., 2000; Loa et al., 2002). Compared with t10, c12-CLA isomer, peripheral blood T lymphocytes culturing with media containing c9, t11-CLA isomer show significantly greater proliferation. These findings suggested the effect of c9, t11-CLA isomer on modulation of peripheral blood...
CLA isomer, c9,t11-CLA isomer had the blood. This finding led to the conclusion that compared which also caused the B cell depletion in peripheral type-2 porcine circovirus-induced immunosuppression, CLA ameliorated viral infectivity in a pig model of similar study, Bassaganya-Riera et al. (2003) reported ens infected with infectious bursal disease virus (Long et al., 2010) and alleviated immunosuppression in chick-chickens under CsA-immunosuppressive status (Long et al., 2010) and alleviated immunosuppression in chickens infected with infectious bursal disease virus (Long et al., 2011) associated with lymphoid depletion. As the similar study, Bassaganya-Riera et al. (2003) reported CLA ameliorated viral infectivity in a pig model of type-2 porcine circovirus-induced immunosuppression, which also caused the B cell depletion in peripheral blood. This finding led to the conclusion that compared with t10,c12-CLA isomer, c9,t11-CLA isomer had the function of alleviating immunosuppression in animals. In the current study, both CLA isomers had the tendency of CsA-induced immunosuppression through increasing the production of IL-2 at the cellular level, and c9,t11-CLA isomer was better than t10,c12-CLA, which is basically consistent with the previous reports.

In T cell receptor signal transduction, a fraction of PLC, a cytosolic protein, is inducibly recompartmentalized to the lipid rafts, which was a part of cell membranes, including important signaling molecules influencing immune function. The PLC-mediated hydrolysis of phosphatidylinositol (4,5)-bisphosphate to inositol (1,4,5)-trisphosphate and diacylglycerol controls Ca2+ mobilization and PKC activation, respectively, critical steps that regulate IL-2 transcription (Barker et al., 1998). In lymphocytes, PLC stimulates IL-2 receptor expression, activates PKC, and promotes cell growth in the presence of IL-2 (Schütze et al., 1994). In this study, both CLA isomers increased the PLC activities of peripheral blood T lymphocyte under CsA-induced immunosuppression, which led to increased the production of IL-2, but it was not significant. The reason may be that the signaling pathway of peripheral blood T lymphocyte was a complex course that was involved in the other pathway including MAPK, IP3/Ca2+, JAK/STAT, and so on (Cantrell, 1996; van der Bruggen et al., 1999; Bocca et al., 2007). In addition, other cytokines, such as TNF-α, IL-1, IL-6, IL-4, IL-10, also had effects on the production of IL-2 (Tomizawa et al., 1991; Narayan et al., 2011; Vinolo et al., 2011). To clarify this, further studies are necessary.

Protein kinase C plays a crucial role in the initial events of signal transduction (di Giacomo et al., 2010), which is important to the activation of T-lymphocyte (Arendt et al., 2002). In naive peripheral T cells, activation of PKC via the T cell receptor is required for IL-2 secretion, IL-2 receptor upregulation, and clonal expansion of CD4 and CD8 T cells (Sakowicz-Burkiewicz et al., 2008). Normal activation of PKC relies on the stimulation of phospholipid hydrolysis that follows a growth factor-receptor interaction. Activated PLC could hydrolyze membrane phosphatidylycholine into diacylglycerol and phosphocholine, and diacylglycerol is known to enhance the PKC activity (Way et al., 2000). Here, we found that CsA induced the decrease of PKC in peripheral blood T lymphocyte. Compared with t10,c12-CLA isomer, c9,t11-CLA isomer significantly increased the production of PKC. This showed that CLA, predominantly the c9, t11-CLA isomer, supplementation enhanced immune function in the CsA-immunosuppressive status in peripheral blood T lymphocyte, which was mediated by the enhancement of PKC.

In conclusion, peripheral blood T lymphocytes in chickens were cultured with media containing 2 CLA isomers, and predominantly the c9, t11-CLA isomer alleviated CsA-induced immunosuppression through increasing lymphocyte proliferation and the production of IL-2. The immune modulatory mechanism by which
c9, t11-CLA had alleviating actions was in part via increasing the signaling molecules such as PLC and PKC.

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