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Effect of Arachidonic Acid on Production of Laminin and Connexin of Granulosa Cells from Chicken Pre-hierarchical Follicles

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ABSTRACT : Arachidonic acid (AA) is a polyunsaturated fatty acid that is a normal constituent of membrane lipids in animal cells. In addition to its role as a precursor of prostaglandins, AA itself may play an important role in the regulation of cell function. The effect of AA on functions of granulosa cells was investigated in pre-hierarchical small yellow follicles of laying hens. Immuno-cytochemical staining showed that AA (10^{-7}-10^{-5} M) increased the expression of the extracellular matrix glycoprotein laminin, gap junction connexin 43 and protein kinase C (PKC). Therefore, mediated by the PKC signal pathway, AA may regulate the intercellular communication of granulosa cells and follicular development by increasing the expression of laminin and connexin. (**Key Words:** Granulosa Cell, Arachidonic Acid, Laminin, Connexin 43, Chicken)

INTRODUCTION

Arachidonic acid (AA) is a polyunsaturated fatty acid (PUFA) that is involved in many cellular signaling mechanisms and is also the precursor for prostaglandin (PG) formation (Sardesai, 1992) that may enhance proliferation of granulosa cells, hence to facilitate development of chicken prehierarchical follicles (Jin et al., 2007). It has been proved that AA markedly affected the expression of genes clustered as: signal transduction pathways, transcription factors, cell cycle, defense and repair, apoptosis, DNA synthesis, cell adhesion, cytoskeleton and related genes (Verlengia et al., 2003; Harizi et al., 2008). Moreover, different levels of metabolites produced from AA by the activity of cytochrome P450 in adipose tissues might be related with the fatness between the Korean native and Yorkshire pigs (Choi et al., 2008).

The extracellular matrix (ECM) played many important roles on cell behaviour, such as migration, division, differentiation, cell death and cell anchorage (Vakonakis and Campbell, 2007). Laminins (LN), a family of ECM glycoproteins, have been implicated in a wide variety of biological processes including cell adhesion, differentiation, migration, signaling, and tumor metastasis (Mecham, 1991; Rodgers et al., 2000). Besides the supporting of the extracellular matrix, intercellular communication is also essential for cell development and functions. Gap junctions are specialized cell-cell junctions that directly link the cytoplasm of neighboring cells. Gap junctions between oocyte and granulosa cells appear to have an important role in the cross talk between the two cell types. Among connexin (Cx) proteins, Cx43 and Cx37 play an important role in ovarian folliculogenesis (Grazul-Bilska et al., 1997).

Development of the prehierarchical follicles is essential for further follicular selection and laying performance in poultry. Dietary supplementations of different natural products are utilized to increase the laying performance. For example, daidzein, a soybean-derived phytoestrogen, can improve development of preovulatory follicles in white silky fowls (a native species) and Isa (a commercial layer) after the peak-laying period (Liu et al., 2007; Liu and Zhang, 2008). ECM and gap junctions play important roles during the development of follicle cells, but up to now, the effect of PG's precursor-AA on ECM, gap junctions and follicle development is still unclear. Therefore, the present study was designed to investigate the effect of AA on granulose cells from prehierarchical small yellow follicle (SYF), with the purpose of increasing laying performance via nutrient regulation.

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MATERIALS AND METHODS

Culture of granulosa cells

The buff laying hens were sacrificed by cervical bleeding post anesthesia. A pool of SYF (6-8 mm in diameter) were removed and placed in ice-cold M199 medium (Hyclone, Utah). After separation from the theca layer, the granulosa sheets were dispersed into the single cells by 12.5 μg/ml collagenase according to a previous method (Jin et al., 2006). Then the cell suspension was seeded in collagen-treated 96-well culture plates (Nunc, Denmark) at a density of 5×10^4/well in 200 μl medium supplemented with 0.5% fetal calf serum (FCS, GIBCO BRL). Cells were incubated at 39°C in a water-saturated atmosphere of 95% air and 5% CO_2.

Treatment of cultured cells

At the beginning of culture, cells were seeded in 0.5% FCS-supplemented medium. After 16 h incubation, the cells attached to the plate bottom and then the medium was replaced with serum-free medium that was supplemented with 10 μg/ml insulin, 5 μg/ml transferrin and 3×10^{-8} M selenite (Sigma, St. Louis, MO, USA). The cells were treated with AA (Sigma) at 10^{-7}-10^{-5} M for 24 h. Chemicals were dissolved in ethanol and diluted with medium. The concentration of ethanol in the medium was ≤0.1%. Controls received the vehicle only.

Immunocytochemistry of LN, Cx43, PKA and PKC

The cultured cells were fixed in 4% neutral paraformaldehyde for 20 min at room temperature and the endogenous peroxidase was quenched with 3% H_2O_2 in PBS for 20 min. Cells were incubated for 20 min with blocking buffer (PBS containing 15% neonate bovine serum) and then with rabbit anti-LN, Cx43 and PKA, monoclonal antibody (Boster Co., Wuhan, China) diluted 1:50 in PBS for 24 h at 4°C. The negative control was prepared in an identical manner except that the primary antibody was replaced with normal PBS. The secondary antibody was goat anti-rabbit IgG. The protocol for PKC immunocytochemistry was similar to that described in PKA immunocytochemistry, except that first antibody was replaced with mouse anti-PKC. Immunoreaction was detected by using SABC system as described in the manufacturer's protocol. Nuclei that were brown to black were counted as the positive cells. The expression levels of

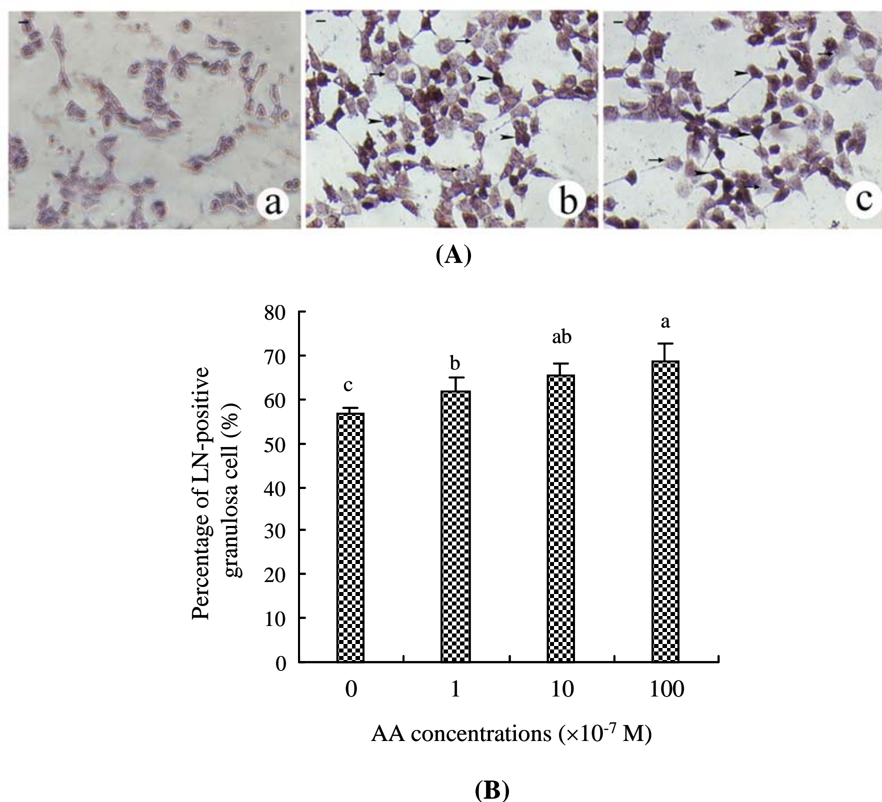


Figure 1. Effects of AA on expression of laminin in granulosa cells after 24 h culture. (A) Immunocytochemical demonstration of laminin expression in granulosa cells. a: negative control; b/c: AA treatment at 0 and 10^{-5} M, respectively. Arrows indicate the negative cells and arrowheads indicate the positive cells. Scale bar: 10 μm. (B) The level of laminin expression in granulosa cells, which was denoted as the ratio of LN-positive cells to the total cell number. Values represent the means±SEM (n = 4). Bars with different superscripts were statistically different at $p<0.05$.

LN, Cx43, PKA or PKC were calculated as the percentages of positively stained cells versus the total cells in the same fields.

Count of cell number

Granulosa cells were observed under a phase contrast microscope. Five different regions were selected randomly in each well and the images were captured with a digital video camera (Pixera Pro 150ES, USA) to a computer. The numbers of granulosa cells were counted by using Simple PCI Advanced Imaging Software (Compix, Inc., USA).

Statistical analysis

The experiment was repeated at least three times and each treatment included 4 wells. All data were expressed as the mean±SEM and evaluated by ANOVA and Duncan's multiple range tests using the SAS 6.12 software. $p<0.05$ was considered significantly different.

RESULTS AND DISCUSSION

Effect of AA on LN expression in granulosa cells

LN is expressed by bovine granulosa cells (Zhao and Luck, 1995). Immunocytochemical study showed that granulosa cells of SYF expressed LN (Figure 1A). When granulosa cells were treated with AA at 10^{-7}-10^{-5} M, the level of LN expression was increased (Figure 1B, $p<0.05$), which suggests LN can be synthesized by granulosa cells and the expression of LN can be strengthened by AA treatment. PUFA have also been shown to influence ECM formation. It has been reported that linoleic acid suppressed collagen production in avian chondrocytes (Watkins et al., 1996), but AA increased the collagen production in porcine medial collateral ligament fibroblasts (Jia and Turek, 2004).

Effect of AA on Cx43 expression in granulosa cells

Cx43 is expressed in porcine granulosa cells following activation of follicular development and this expression significantly increased throughout follicular growth and maturation (Melton et al., 2001). In this study, the expression of Cx43 in granulosa cells of chicken SYF can be strengthened by AA (Figure 2A) and it showed ascending tendency in a dose-dependent manner (Figure 2B, $p<0.05$). Cx43 is believed mainly to be a granulose cell gap junction protein within the ovarian follicles (Risek et al., 1990). Knockout study shows that Cx43 is essential for

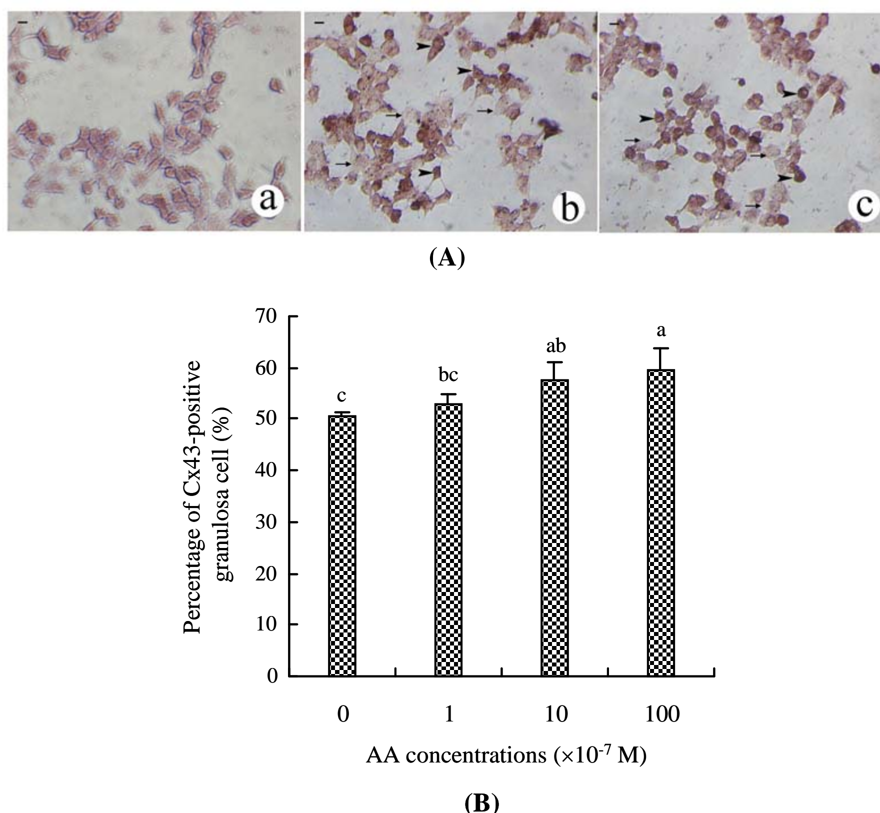
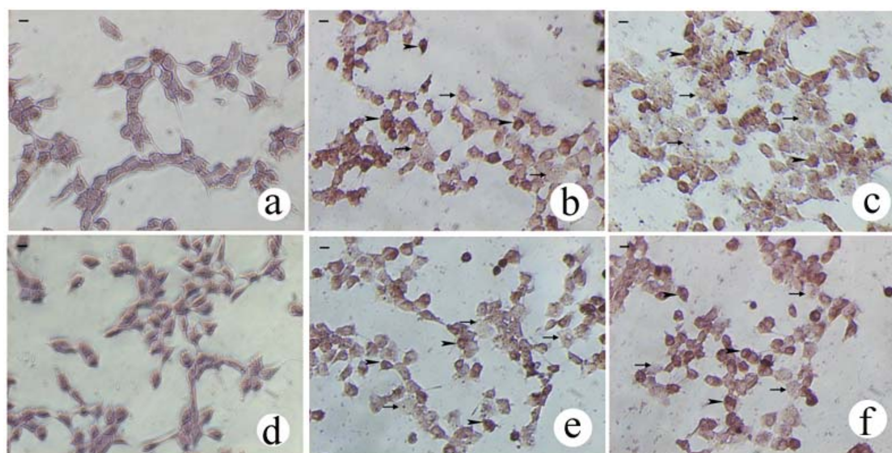


Figure 2. Effects of AA on expression of Cx43 in granulosa cells after 24 h culture. (A) Immunocytochemical demonstration of Cx43 expression. a: negative control; b/c: AA treatment at 0 and 10^{-6} M for Cx43, respectively; Arrows indicate the negative cells and arrowheads indicate the positive cells. Scale bar: 10 µm. B: The level of Cx43 expression in granulosa cells, which was denoted as the ratio of Cx43-positive cells to the total cell number. Values represent the means±SEM (n = 4). Bars with different superscripts were statistically different at $p<0.05$.

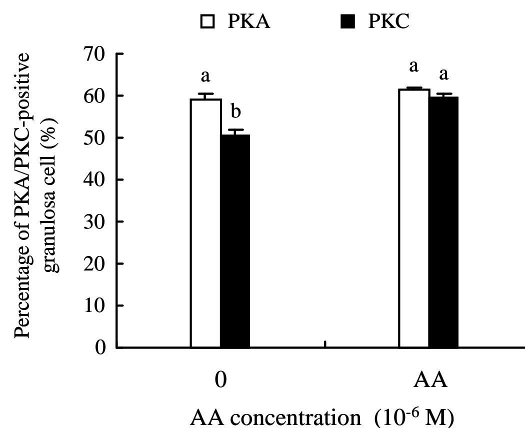
normal follicle growth in the mouse. In fact, the absence of Cx43 affects folliculogenesis by arresting follicle growth at early stages of development (Juneja et al., 1999). This defective granulosa cell development leads to severe consequences for the developing oocytes that fail to undergo meiotic maturation (Ackert et al., 2001). Therefore, it can be presumed that AA increased expression of Cx43 to mediate the exchange of nutriment and signaling between granulosa cell and oocyte to promote the follicle development.

Effect of AA on PKA and PKC expression in granulosa cells

AA directly affects the functional state of various molecules, including the protein kinase C (PKC) (Lester et al., 1991) and calcium channels (Mochizuki-Oda et al., 1993). Our immunocytochemical studies showed that granulosa cells were positive for PKA when cells received only vehicle. AA treatment did not significantly increase PKA expression in granulosa cells (Figure 3A and B, $p<0.05$). By contrast, after treatment with AA (10^{-7}-10^{-5} M), the granulosa cells showed more intense staining for PKC than the control (Figure 3A) and the ratio of PKC-positive cells increased in a dose-dependent manner (Figure 3B, $p<0.05$). It is concluded that AA is mainly mediated by PKC signal pathway to influence the granulosa cells of SYF. A previous (Hertelendy et al., 1992) study showed that AA plays an important role in signal transduction in avian granulosa cells by PKC signal pathway. It has been proved (Kariya and Miyazaki, 2004) that epithelial cells at wound edges or carcinoma cells could synthesize LN5. Then LN5 induces PKC-dependent signaling, leading to the stimulation of cell migration. Therefore, by promoting LN expression in granulosa cells of SYF to induce PKC signal



(A)



(B)

Figure 3. Effects of AA on expression of PKA and PKC in granulosa cells after 24 h culture. (A) Immunocytochemical demonstration of PKA and PKC expression. a: negative control; b/c: AA treatment at 0 and 10^{-6} M for PKA, respectively; d: negative control; e/f: AA treatment at 0 and 10^{-6} M for PKC, respectively. Arrows indicate the negative cells and arrowheads indicate the positive cells. Scale bar: 10 μm. (B) The level of PKA/PKC expression in granulosa cells, which was denoted as the ratio of PKA/PKC-positive cells to the total cell number. Values represent the means±SEM (n = 4). Bars with different superscripts were statistically different at $p<0.05$.

pathway, AA may lead to cell migration throughout oogenesis during follicular development.

There exists cross talk between different cellular signaling pathways. PKC has for example been shown to activate the MAPK through phosphorylation of RAF (Kolch et al., 1993). Cx43 is originally synthesized as the non-phosphorylated form and is then inserted in the plasma membrane to convert into the phosphorylated forms. Phosphorylated Cx43 is thought to be the main functional form of Cx43 (Musil and Goodenough, 1991). It has been proved that PKC can regulate gap junctional communication by phosphorylated connexin43 on serine 368 (Lampe et al., 2000). Therefore, by PKC signal pathway, AA can increase the Cx43 mRNA and protein levels and by phosphorylation of connexin 43 to regulate the communication between oocyte and granulosa cell and follicle development.

In conclusion, mediated by PKC signaling pathway, AA can regulate intercullular communication of follicular cells and development through increasing the production of ECM glycoprotein LN and gap junction protein Cx43 in the granulosas cells of prehierachical follicles in laying hens.

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