PHYSIOLOGY

Leukemia Inhibitory Factor Decreases the Arylamine N-Acetyltransferase Activity in Human Cumulus Granulosa Cells

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Purpose: To evaluate the activities of acetyl coenzyme A (AcCoA): arylamine N-acetyltransferase (NAT) of intact cumulus granulosa cells and the role of leukemia inhibitory factor (LIF) upon their NAT activities.

Methods: Thirty women accepted controlled ovarian hyperstimulation (COH) and oocyte retrievals. Human cumulus granulosa cells were obtained during oocyte retrievals. Using 2-aminofluorene (2-AF) and p-aminobenzoic acid (PABA) as substrates, NAT activity of all samples was determined by high pressure liquid chromatography. After the incubation with different time and concentrations of 2-AF, PABA, and LIF, 2-acetyl-aminofluorene (2-AAF) and N-acetyl-PABA (N-Ac-PABA) were measured.

Results: After incubation with 2.812, 5.625, 11.25, and 22.5 μM of 2-AF/PABA, their product concentrations (2-AAF/N-Ac-PABA) were 0.42/0.32, 0.76/0.58, 1.29/1.04, and 1.94/1.26 nmol/ 10^6 cells, respectively. After 6, 12, 18, and 24 h incubation with 11.25 μM of 2-AF/PABA, their product concentrations were 0.19/0.12, 0.56/0.4, 0.98/0.79, and 1.3/1.0 nmol/ 10^6 cells, respectively. After incubation with 0, 5, and 50 μM of LIF, the 2-AAF/N-Ac-PABA concentra-

tions were 0.98/0.80, 0.70/0.52, and 0.49/0.30 nmol/ 10^6 cells, respectively.

Conclusion: Intact human cumulus granulosa cells could acetylate arylamine carcinogen (2-AF) and noncarcinogens drug (PABA). LIF decreased the NAT activities. It provides a model for monitoring the effects of COH and LIF upon the oocytes.

KEY WORDS: 2-aminofluorene; cumulus cell; leukemia inhibitory factor; N-acetyltransferase; ovarian hyperstimulation.

INTRODUCTION

N-acetyltransferase (NAT) activity is involved in the detoxification of exogenous amines. Both N-acetylation and deacetylation reactions are important in the activation of carcinogenic arylamine to their nontoxic and toxic forms. The capacity to acetylate amino-containing compounds may determine an individual's predisposition to toxicity from several agents (1). The carcinogenesis of the colon and bladder are related with the phenotypic abilities of the individual to N-acetylation arylamine carcinogens (2,3). It has been reported that extrahepatic expression of acetylator-genotype dependent NAT activity occurs in human colon (2) and bladder (3).

The relationship between fertility drugs with the increasing ovarian tumors have been demonstrated (4,5). However, there is no available data about the NAT activity in human cumulus. In another one of our studies, we observed that the cytoplasm of human cumulus granulosa cells could acetylate arylamine

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carcinogen (2-aminofluorene, 2-AF) and noncarcinogens drug (PABA) (6). In this series, by using the intact human cumulus granulosa cells instead of its cytoplasm, we tried to evaluate their NAT activity in vitro. Furthermore, leukemia inhibitory factor (LIF) plays an important role in the embryo development (7–9). We also evaluated the effect of LIF upon the NAT activities. This is the first report in this aspect.

MATERIALS AND METHODS

A total of 30 females who accepted controlled ovarian hyperstimulation (COH) for in vitro fertilization in our unit were included. Using 2-AF as a carcinogen and PABA as noncarcinogen, we tried to evaluate the NAT activity of intact cumulus granulosa cells in vitro. Arylamine NAT activity toward 2-AF and PABA was detected in all 30 human cumulus tissue. The protocol was approved by the Ethical Committee of the China Medical College Hospital. Informed consent was obtained from all couples who were enrolled in this series.

Ovarian stimulation protocol for in vitro fertilization (IVF) using gonadotrophin-releasing hormone agonist (GnRH-a) and gonadotrophins was as previously described (10). In brief, the ovaries were stimulated by follicle stimulating hormone (FSH, Metrodin; Serono, Rome, Italy) or human menopausal gonadotrophin (HMG, Pergonal; Serono, Rome, Italy), and with the long protocol down-regulation of the GnRH-a suppression (Leuprolide acetate depot, Takeda Chemical Industries, Japan) from the mid-luteal phase (menstrual day 21–23). Individualized injection of gonadotrophin was continued until there were two or more follicles ≥ 18 mm, then the human chorionic gonadotrophin (HCG, 10000 IU, Profasi; Serono) was administered. Oocytes were retrieved transvaginally 34–36 h later. The cumulus granulosa cells with Hepes-buffered human tubal fluid medium were obtained during oocyte retrievals and loaded into the Eppendorf tube (1.5 mL).

Cumulus granulosa cells were placed into 75 cm² tissue culture flasks and grown at 37°C under a humidified 5% CO₂ atmosphere in RPMI 1640 medium (Sigma) supplemented with 10% foetal bovine serum (FBS) (Gibco BRL, Grand Island, NY), 2% penicillin–streptomycin (10,000 U/mL penicillin and 10 mg/mL streptomycin). About 5×10^7 human cumulus granulosa cells were placed in 2 mL of the lysis buffer (Tris/HCL (20 mM, pH 7.5, 4°C), acetyl-carnitine, dithiothreitol (1 mM, DTT), Ethylenedi-

aminetetraacetic acid (1 mM, EDTA), 50 μ M PMSF, and 10 μ M leupeptin) (11). The homogenates were centrifuged at 9000g for 1 min in a model 3200 Eppendorf/Brinkman centrifuge (4°C), and the supernatant fraction was subsequently centrifuged at 10,000g for 60 min at 4°C. The supernatant was kept on ice for the NAT activity determinations.

AcCoA-dependent *N*-acetylation of 2-AF and PABA was determined as previously described (11,12). NAT activity was expressed as acetylated substrate (nmol) per min per 10⁶ cumulus granulosa cells. 2-acetyl-aminofluorene (2-AAF) and *N*-acetyl-PABA (*N*-Ac-PABA) were measured by high pressure liquid chromatography (HPLC) (Beckman detector 168, pump 126). An aliquot of the NAT incubation was injected onto a C18 reversed-phase column (Spherisorb 4.6 $\times$ 250 mm²) of a Beckman HPLC (pump 168 and detector 126) and eluted at a flow rate of 1.2 mL/min. For PABA and *N*-Ac-PABA, the solvent system was 50 mM acetic acid/CH₃CN (86:14; v/v) with detection at 266 nm. The retention time for PABA was 8 min and 11 min for *N*-Ac-PABA. For 2-AF and 2-AAF, the solvent system was 20 mM KH₂PO₄ (pH 4.5)/CH₃CN (53:47; v/v) with detection at 280 nm. The retention time was 6.5 min for 2-AAF and 9 min for 2-AF.

All compounds were quantified by comparison of the integrated area of the elution peak with that of known amounts of standards. The reaction mixtures were incubated at 37°C for 10 min and stopped with 100 μ l of acetonitrile for the 2-AF reactions and 50 μ l of 20% trichloroacetic acid (TCA) for the PABA reactions. For single point activity measurements, the final concentration of 2-AF or PABA was 0.1 mM and that of AcCoA was 0.5 mM. All samples were assayed in triplicate. EDTA, Tris, PABA, *N*-acetyl-PABA (*N*-Ac-PABA), dimethyl sulfoxide (DMSO), leupeptin, bovine serum albumin (BSA), phenylmethylsulfonylfluoride (PMSF), DTT, and carnitine acetyltransferase were obtained from Sigma (St. Louis, MO). AcCoA was obtained from P-L Biochemicals (Milwaukee, WI). 2-AF and 2-AAF were obtained from K and K Laboratories (Plainview, NY).

Human cumulus granulosa cells (in 1 mL RPMI 1640 media with glutamine and 10% FBS) were incubated with 2-AF and PABA (11.25 μ M) at 1×10^6 cells/mL in individual wells of 24-well cell culture plate after incubation (37°C, 95% air/5% CO₂, 18 h) with different concentrations of LIF (0, 5, 60 μ M) (13). The cells and media were removed and centrifuged to determine the NAT activity. For experiments with

2-AF, the supernatant was immediately extracted with ethyl acetate/methanol (95:5), the solvent evaporated, and the residue redissolved in methanol and assayed for 2-AAF as described above. For experiments with PABA, aliquots of the supernatant were assayed directly for *N*-Ac-PABA. The SAS system with unpaired student's *t*-test was utilized for statistical analyses. A *p*-value <0.05 was considered statistically significant.

RESULTS

Acetylated products (2-AAF, *N*-Ac-PABA) of cumulus granulosa cells with different concentrations of 2-AF and PABA (2.812, 5.625, 11.25, and 22.5 mM) are represented in Fig. 1. Overall, the 2-AF NAT activity was higher than the PABA-NAT activity. The data indicated that the NAT activity increased with increased concentrations of 2-AF and PABA. After incubation with 2.812, 5.625, 11.25, and 22.5 μ M of 2-AF and PABA, the 2-AAF/*N*-Ac-PABA concentrations were $0.42 \pm 0.08/0.32 \pm 0.06$, $0.76 \pm 0.1/0.58 \pm 0.08$, $1.29 \pm 0.2/1.04 \pm 0.14$, and $1.94 \pm 0.42/1.26 \pm 0.26$, respectively (Fig. 1).

The NAT activities with different period of incubation are given in Fig. 2. The NAT activities increased with increased incubation period of 2-AF and PABA. After 6, 12, 18, and 24 h incubation with 11.25 μ M of 2-AF and PABA, 2-AAF/*N*-Ac-PABA concentrations were $0.19 \pm 0.08/0.12 \pm 0.04$, $0.56 \pm 0.12/0.4 \pm 0.08$, $0.98 \pm 0.18/0.79 \pm 0.12$, and $1.3 \pm 0.22/1.0 \pm 0.16$, respectively. The NAT activities decreased with increased concentrations of LIF. After incubation with 0, 5, and 50 μ M of LIF, the 2-AAF/*N*-Ac-PABA

Fig. 1. Acetylated products (2-AAF, *N*-Ac-PABA) of cumulus cells after 18 h incubation with different concentrations of AF and PABA (2.812, 5.625, 11.25, and 22.5 mM).

Fig. 2. Acetylated products (2-AAF, *N*-Ac-PABA) of cumulus cells after 6, 12, 18, and 24 h incubation with AF and PABA (11.25 mM).

concentrations were $0.98 \pm 0.20/0.80 \pm 0.16$, $0.70 \pm 0.18/0.52 \pm 0.12$, and $0.49 \pm 0.18/0.30 \pm 0.08$, respectively (Table I).

DISCUSSION

Relationship between COH and ovarian carcinogenesis is controversial (5,14). Some investigators demonstrated the relationship between COH and the increased risk of ovarian tumors (4,5). However, there was no logic method or model to evaluate the ovarian status during COH. NAT activities are related with carcinogenesis (15). NAT plays a major role in the detoxification of exogenous amines (16). The genetically mediated variation in NAT activities within target organs or tissues for arylamine-induced neoplasm may indicate different risks among human populations. Because the ovaries are the target organ of COH, it is reasonable to speculate that cumulus granulosa cells may be involved in the acetylation of arylamine carcinogens.

NAT exists in different forms in various species and tissues (17,18). Numerous reports about NAT in human organs have been demonstrated, including its activity in the umbilical cord (19), placenta (11), liver (16), breast (20), small intestine (21), colon (2),

Table I. *N*-Acetyltransferase Activities of Human Cumulus Granulosa Cells at Various Concentrations of LIF

LIF (μ M)	2-AAF (nmol/ 10^6 cells)	<i>N</i> -Acetyl-PABA (nmol/ 10^6 cells)
0	$0.98 \pm 0.20^*$	0.80 ± 0.16
5	$0.70 \pm 0.18^*$	$0.52 \pm 0.12^*$
50	$0.49 \pm 0.18^*$	$0.30 \pm 0.08^*$

* *p* < 0.05.

lung (22), kidney (23), bladder (3), and prostate (23). However, there are no available reports about the acetylation of intact cumulus granulosa cells toward a carcinogen (2-AF) and a noncarcinogen (PABA). In another one of our studies, we observed that there was a higher percentage of rapid acetylators established in the cytoplasm of human cumulus granulosa cells (6). In this series, we established the preliminary database about the NAT activities of intact ovarian cumulus granulosa cells during COH. We observed that the NAT activity increased with increased concentrations of 2-AF/PABA and their prolonged incubation. However, the role of arylamine NAT activity in the cumulus granulosa cells remains unknown although its usefulness in metabolism of endogenous amines is obvious.

LIF plays a role in human reproduction (7–9). In our previous series, we observed that the LIF enhances the development of preimplantation human and mouse embryos in vitro (7–9,24). van Eijk *et al.* (25) demonstrated that LIF receptor (LIF receptor beta, glycoprotein 130) presented in the oocyte and early stage of embryos. Ozornek *et al.* (26) demonstrated the LIF could be detected in follicular fluid. In this series, we evaluated that the NAT activities decreased with increased concentrations of LIF. This suggested that the LIF or other cytokines might play a role in the processes of *N*-acetylation, detoxification, and follicular growth.

Rapid and slow acetylations have been demonstrated as a predisposing factor for the sensitivity of individuals to the toxicity during exposure to many arylamines (27). It has been reported that rapid acetylators are related with a higher risk of colorectal cancer (28), and slow acetylators is related with bladder cancer (3). An enhanced *N*-acetylation capacity is related with developing colon cancer; an inactive *N*-acetylation is associated with developing bladder tumors.

In conclusion, this study provided the preliminary database about NAT activity in human intact cumulus granulosa cells. Intact human cumulus granulosa cells could acetylate arylamine carcinogen (2-AF) and noncarcinogens drug (PABA). LIF decreased the NAT activities. It provides a model for monitoring the effects of COH, LIF, and other cytokines upon the oocytes. However, the real roles of NAT activity and LIF upon the ovarian status during COH merits further investigation. Furthermore, the influences of other cytokines upon the NAT activity deserve further surveys.

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