PHYSIOLOGY

Kinetics of Acetyl Coenzyme A: Arylamine N-Acetyltransferase from Human Cumulus Cells

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Purpose: N-acetyltransferase (NAT) activity is involved in the detoxification of exogenous amines. We aimed to evaluate the kinetics of acetyl coenzyme A (AcCoA): arylamine NAT for human cumulus cells.

Methods: Thirty infertile women who were undergoing controlled ovarian hyperstimulation (COH) and oocyte retrievals were recruited. Human cumulus cells were obtained during oocyte retrievals. Using 2-aminofluorene (2-AF) and p-aminobenzoic acid (PABA) as substrates, NAT activity and Michaelis-Menten kinetics constants of all samples were determined by using high-pressure liquid chromatography.

Results: There were 6 rapid, 10 intermediate, and 14 slow acetylators. 2-AF-NAT and PABA-NAT activities were 0.97 ± 0.74 and 0.89 ± 0.77 nmol/min/mg protein, respectively. K_m/V_{max} of rapid and slow acetylators for 2-AF were $(161 \pm 55)/(15.6 \pm 2.9)$ and $(27.8 \pm 11.4)/(2.6 \pm 0.9)$, respectively. K_m/V_{max} of rapid and slow acetylators for PABA were $(104 \pm 36)/(13.2 \pm 2.8)$ versus $(20.0 \pm 10)/(2.0 \pm 0.7)$, respectively. Compared to slow acetylators, the rapid acetylators exhibited higher K_m/V_{max} values for 2-AF (5.8-/6-fold) and PABA (6-/6.6-fold), respectively.

Conclusion: Human cumulus could acetylate arylamine carcinogen (2-AF) and noncarcinogen drug (PABA). Higher percentage of rapid acetylators established in the cumulus during COH. It provides a model for monitoring the effects of pollution or carcinogenesis upon the oocyte during COH and oocyte retrievals.

KEY WORDS: 2-aminofluorene; cumulus cell; N-acetyltransferase; NAT; ovarian hyperstimulation; p-aminobenzoic acid.

INTRODUCTION

N-acetyltransferase (NAT) activity is involved in the detoxification of exogenous amines. N-acetylation, a major metabolic pathway of arylamines carcinogens, is catalyzed by cytosolic arylamine NAT, using acetyl coenzyme A (AcCoA) as a cofactor. Both N-acetylation and deacetylation reactions are important in the activation of carcinogenic arylamine to its non-toxic and toxic forms. The capacity to acetylate amino-containing compounds may determine an individual's predisposition to toxicity from several agents (1). In humans, 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 of fertility drugs with the increasing ovarian tumors has been demonstrated (4). However, there is no available data about the NAT activity in human cumulus cells. Recent studies have demonstrated that the NAT activity in the

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placentas was based on both monomorphic (PABA) and polymorphic (SMZ) as NAT substrate (5) and carcinogen (2-aminofluorene) (6). In this series, using 2-aminofluorene (2-AF) as a carcinogen and *p*-aminobenzoic acid (PABA) as noncarcinogen, we tried to evaluate the NAT activity of cumulus cells in vitro. This is the first report about the NAT activity in human cumulus cells.

MATERIALS AND METHODS

A total of 30 females who accepted controlled ovarian hyperstimulation (COH) for in vitro fertilization (IVF) in our unit were included. 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 signed by all couples who were enrolled in this series.

Ovarian stimulation protocol for in vitro fertilization, using gonadotrophin-releasing hormone agonist (GnRH-a) and gonadotrophins was as previously described (7). 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 midluteal 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, 10,000 IU, Profasi; Serono) was administered. Oocytes were retrieved transvaginally 34–36 h later. The cumulus cells with Hepes-buffered human tubal fluid medium were obtained during oocyte retrievals and loaded into the Eppendorf tube (1.5 mL).

Individual cumulus cells were placed in 5 volumes of the lysis buffer [Tris/HCL (20 mM, pH 7.5, 4°C), acetylcarnitine, dithiothreitol (1 mM, DTT), ethylenediaminetetraacetic acid (1 mM, EDTA), 50 μ M phenyl methyl sulfonyl fluoride (PMSF), and 10 μ M leupeptin] (6). The tissues were homogenized on ice with a polytron homogenizer set (PT-MR 3000, Kinematica AG, Littau-Switzerland) at 25,000 rpm for 20 s (twice). The homogenates were centrifuged at $9,000 \times g$ for 15 min in a model 3200 Eppendorf/Brinkman centrifuge (4°C), and the supernatant fraction was subsequently centrifuged at $100,000 \times g$ for

60 min at 4°C. The supernatant was kept on ice for the NAT activity and protein determinations. EDTA, Tris, PABA, *N*-acetyl-PABA (*N*-Ac-PABA), dimethyl sulfoxide (DMSO), leupeptin, bovine serum albumin (BSA), 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-acetyl-aminofluorene (2-AAF) were obtained from K and K Laboratories (Plainview, NY).

Using 2-AF and PABA as substrates, NAT activity and Michaelis-Menten kinetic constants were determined by high pressure liquid chromatography (HPLC) (Beckman detector 168, pump 126). AcCoA-dependent *N*-acetylation of 2-AF and PABA was determined as previously described (6,8). Incubation mixtures (90 μ L) consisted of tissue cytosol in lysis buffer (50 μ L, 20 mM Tris/HCl, pH 7.5, 1 mM DTT and 1 mM EDTA), AcCoA (20 μ L), and AcCoA recycling mixture [20 μ L, 50 mM Tris/HCl (pH 7.5), 0.2 mM EDTA, 2 mM DTT, 15 mM acetylcarnitine, 2 U/mL carnitine acetyltransferase, 2-AF or PABA at the specific concentration]. 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. 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. All samples were assayed with triplicate.

After centrifugation, the amounts of acetylated product and remaining nonacetylated substrate were determined by HPLC analysis (6,8). An aliquot of the NAT incubation was injected onto a C18 reversed-phase column (Spherisorb 4.6 $\times$ 250 mm) 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; vol/vol) with detection at 266 nm. The retention time was 8 min for PABA 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; vol/vol) 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.

Using 1.0 and 2.0 nmol/min/mg protein as the dividing line, NAT activities of 30 samples were divided into three groups: rapid (≥ 2.0 nmol/min/mg protein), intermediate (< 2.0 , ≥ 1 nmol/min/mg protein),

Table I. *N*-Acetyltransferase Activity of Slow, Intermediate, and Rapid Acetylators Phenotypes in Tissue Homogenates from Human Cumulus Tissues

Patient's no.	2-AAF (nmol/min/mg protein)	<i>N</i> -Acetyl-PABA (nmol/min/mg protein)
Rapid (<i>n</i> = 6)		
1	2.20 ± 0.34	2.06 ± 0.23
2	2.16 ± 0.26	2.12 ± 0.22
3	2.12 ± 0.22	2.10 ± 0.20
4	2.08 ± 0.24	2.08 ± 0.22
5	2.06 ± 0.28	2.05 ± 0.19
6	2.01 ± 0.22	2.00 ± 0.14
Mean	2.10 ± 0.22 ^a	2.08 ± 0.20 ^b
Intermediate (<i>n</i> = 10)		
7	1.80 ± 0.12	1.76 ± 0.14
8	1.52 ± 0.18	1.32 ± 0.12
9	1.32 ± 0.10	1.14 ± 0.10
10	1.20 ± 0.12	1.13 ± 0.14
11	1.16 ± 0.08	1.10 ± 0.10
12	1.12 ± 0.12	1.09 ± 0.12
13	1.10 ± 0.10	1.07 ± 0.06
14	1.06 ± 0.04	1.04 ± 0.03
15	1.02 ± 0.12	1.02 ± 0.10
16	1.00 ± 0.04	1.00 ± 0.06
Mean	1.23 ± 0.10 ^{a,c}	1.16 ± 0.08 ^{b,d}
Slow (<i>n</i> = 14)		
17	0.82 ± 0.22	0.86 ± 0.13
18	0.54 ± 0.20	0.46 ± 0.10
19	0.48 ± 0.12	0.36 ± 0.09
20	0.40 ± 0.12	0.23 ± 0.08
21	0.38 ± 0.10	0.18 ± 0.06
22	0.36 ± 0.12	0.14 ± 0.08
23	0.32 ± 0.10	0.10 ± 0.04
24	0.24 ± 0.10	0.09 ± 0.06
25	0.20 ± 0.10	0.08 ± 0.04
26	0.18 ± 0.06	0.07 ± 0.03
27	0.12 ± 0.04	0.06 ± 0.03
28	0.10 ± 0.04	0.06 ± 0.02
29	0.08 ± 0.04	0.04 ± 0.02
30	0.06 ± 0.02	0.02 ± 0.01
Mean	0.30 ± 0.09 ^c	0.19 ± 0.11 ^d
Total	0.97 ± 0.74	0.89 ± 0.77

Note. Values are mean ± SD. Cytosols were incubated with 2.25 mM AF or PABA for 10 min. NAT activity is expressed as 2-AAF or N-AC-PABA nmol/min/mg protein of cytosolic protein. Superscript symbols^{a,c} indicate significant difference in the 2-AAF activity ($p < .005$, $p < .01$, respectively); Superscript symbols^{b,d} indicate significant difference in the *N*-Acetyl-PABA activity ($p < .001$, $p < .05$, respectively).

and slow (<1.0 nmol/min/mg protein) activity groups (Table I). K_m/V_{max} values of three groups were determined. The kinetic constants were calculated with the Cleland HYPER Program (9). Acetylation catalyzed by NAT follows ping-pong kinetics (10), so that increasing the concentration of AcCoA in a system containing a standard concentration of the acetylators arylamine will increase both apparent K_m and V_{max} values of the arylamine substrate. The different K_m/V_{max} values could indicate a second NAT isoform with different kinetic properties. The SAS system with unpaired student's *t* test was utilized for statistical analyses. A *p*-value <.05 was considered statistically significant.

RESULTS

There were 6, 10, and 14 samples in the rapid, intermediate, and slow activity acetylators, respectively (Table I). Overall, the 2-AF NAT activity was higher than the PABA-NAT activity. Their activities were 0.97 ± 0.74 and 0.89 ± 0.77 nmol/min/mg protein for 2-AF and PABA, respectively. The activities in the rapid, intermediate, and slow acetylators for 2-AF were 2.10 ± 0.22 , 1.23 ± 0.10 , and 0.30 ± 0.09 nmol/min/mg protein, respectively. The activities in the rapid, intermediate, and slow acetylators for PABA were 2.08 ± 0.20 , 1.16 ± 0.08 , and 0.19 ± 0.11 nmol/min/mg protein, respectively (Table I).

Table II. Kinetic Data for Arylamine of Rapid, Intermediate, and Slow *N*-Acetyltransferase in Human Cumulus Tissues

Patient's no.	Apparent Km		Apparent $V_{\max}$	
	AF	PABA	AF	PABA
Rapid ($n = 6$)				
1	190 $\pm$ 70	115 $\pm$ 40	22.6 $\pm$ 4.2	14.2 $\pm$ 3.0
2	180 $\pm$ 60	110 $\pm$ 40	20.6 $\pm$ 3.2	14.0 $\pm$ 3.0
3	180 $\pm$ 60	120 $\pm$ 50	21.6 $\pm$ 3.4	14.4 $\pm$ 3.2
4	160 $\pm$ 60	110 $\pm$ 40	17.9 $\pm$ 2.6	12.6 $\pm$ 2.4
5	140 $\pm$ 40	90 $\pm$ 30	16.0 $\pm$ 2.2	13.4 $\pm$ 2.8
6	120 $\pm$ 40	80 $\pm$ 20	15.4 $\pm$ 2.2	10.4 $\pm$ 2.4
Mean	161 $\pm$ 55 ^a	104 $\pm$ 36 ^b	15.6 $\pm$ 2.9 ^c	13.2 $\pm$ 2.8 ^d
Intermediate ($n = 10$)				
7	90 $\pm$ 30	50 $\pm$ 20	10.8 $\pm$ 1.8	8.2 $\pm$ 1.8
8	80 $\pm$ 30	50 $\pm$ 20	9.4 $\pm$ 1.6	7.6 $\pm$ 1.4
9	80 $\pm$ 20	60 $\pm$ 20	8.2 $\pm$ 1.4	7.0 $\pm$ 2.0
10	70 $\pm$ 30	60 $\pm$ 30	8.0 $\pm$ 1.6	6.6 $\pm$ 1.8
11	70 $\pm$ 20	50 $\pm$ 20	7.4 $\pm$ 1.2	6.4 $\pm$ 1.6
12	70 $\pm$ 30	50 $\pm$ 30	7.0 $\pm$ 1.4	6.0 $\pm$ 1.2
13	60 $\pm$ 30	50 $\pm$ 20	6.8 $\pm$ 1.3	5.2 $\pm$ 1.4
14	60 $\pm$ 20	50 $\pm$ 20	6.4 $\pm$ 1.2	5.0 $\pm$ 1.1
15	50 $\pm$ 20	50 $\pm$ 20	5.2 $\pm$ 1.1	5.4 $\pm$ 1.4
16	50 $\pm$ 10	50 $\pm$ 10	4.9 $\pm$ 1.2	5.6 $\pm$ 1.6
Mean	68.0 $\pm$ 22	49 $\pm$ 21	7.4 $\pm$ 1.3	6.3 $\pm$ 1.5
Slow ($n = 14$)				
17	40 $\pm$ 20	20 $\pm$ 10	4.0 $\pm$ 1.2	3.3 $\pm$ 1.2
18	40 $\pm$ 20	20 $\pm$ 10	3.8 $\pm$ 1.2	3.2 $\pm$ 1.2
19	40 $\pm$ 10	20 $\pm$ 10	3.3 $\pm$ 1.0	3.0 $\pm$ 1.0
20	40 $\pm$ 10	20 $\pm$ 10	3.2 $\pm$ 1.0	2.6 $\pm$ 1.0
21	30 $\pm$ 10	20 $\pm$ 10	3.4 $\pm$ 0.9	2.3 $\pm$ 0.8
22	30 $\pm$ 10	20 $\pm$ 10	3.2 $\pm$ 0.8	2.3 $\pm$ 0.6
23	30 $\pm$ 10	20 $\pm$ 10	3.0 $\pm$ 0.9	2.1 $\pm$ 0.8
24	20 $\pm$ 10	20 $\pm$ 10	2.8 $\pm$ 0.8	2.0 $\pm$ 0.6
25	20 $\pm$ 10	20 $\pm$ 10	2.4 $\pm$ 0.6	1.8 $\pm$ 0.6
26	20 $\pm$ 10	20 $\pm$ 10	2.4 $\pm$ 0.8	2.0 $\pm$ 0.6
27	20 $\pm$ 10	20 $\pm$ 10	2.2 $\pm$ 0.6	2.0 $\pm$ 0.6
28	20 $\pm$ 10	20 $\pm$ 10	2.0 $\pm$ 0.8	2.0 $\pm$ 0.6
29	20 $\pm$ 10	20 $\pm$ 10	1.8 $\pm$ 0.6	1.6 $\pm$ 0.5
30	20 $\pm$ 10	20 $\pm$ 10	1.4 $\pm$ 0.4	1.0 $\pm$ 0.3
Mean	27.8 $\pm$ 11.4 ^a	20.0 $\pm$ 10 ^b	2.6 $\pm$ 0.9 ^c	2.0 $\pm$ 0.7 ^d
Total	68 $\pm$ 53	47.5 $\pm$ 32.9	7.6 $\pm$ 6.4	5.8 $\pm$ 4.3

Note. Kinetic data are calculated from the modified HYPER program of Cleland (9). Determined from 100,000 $\times$ g supernatant fractions: experiments performed in triplicate. Values are mean $\pm$ SD. Superscript symbols^{a,b} indicate significant difference in the Km values ($p < .05$); Superscript symbols^{c,d} indicate significant difference in the $V_{\max}$ values ($p < .05$).

Overall Km/ $V_{\max}$ for 2-AF and PABA were (68 $\pm$ 53)/(7.6 $\pm$ 6.4) and (47.5 $\pm$ 32.9)/(5.8 $\pm$ 4.3), respectively (Table II). The rapid activity tissues have higher Km/ $V_{\max}$ values compared to the tissues with intermediate or low activity. The Km/ $V_{\max}$ of rapid, intermediate, and slow acetylators for 2-AF were (161 $\pm$ 55)/(15.6 $\pm$ 2.9), (68.0 $\pm$ 22)/(7.4 $\pm$ 1.3), and (27.8 $\pm$ 11.4)/(2.6 $\pm$ 0.9), respectively. The Km/ $V_{\max}$ of rapid, intermediate, and slow acetylators for PABA were (104 $\pm$ 36)/(13.2 $\pm$ 2.8), (49 $\pm$ 21)/(6.3 $\pm$ 1.5), and (20.0 $\pm$ 10)/(2.0 $\pm$ 0.7), respectively (Table II). Compared to the enzymes from the slow acetylators, the rapid acetylators exhibited higher Km/ $V_{\max}$ val-

ues for 2-AF (5.8-/6-fold) and PABA (6-/6.6-fold), respectively.

Kinetic constants for 2-AAF and *N*-Acetyl-PABA NAT activities with varying concentrations of Ac-CoA were listed in Table III. Selected AcCoA concentrations reported in in vitro liver cytosol were close to 100 mM and the rapid enzyme displayed a Km of 80 mM for PABA (11). We noted that as the AcCoA concentration in the reaction mixture increased, Km/ $V_{\max}$ values for AF and PABA became appreciably higher for rapid, intermediate, and slow isozymes (data not shown).

Table III. Kinetic Constants of 2-AF and PABA NAT Activities of Human Cumulus Tissues at Various Fixed Concentrations of Acetyl CoA

Acetyl CoA	2-AAF		N-Acetyl-PABA	
	Km (mM)	$V_{\max}$ (nmol/min/mg protein)	Km (mM)	$V_{\max}$ (nmol/min/mg protein)
100	16 ± 4	1.4 ± 0.5	14 ± 4	1.2 ± 0.4
125	24 ± 6	2.8 ± 0.8	22 ± 6	2.2 ± 0.8
250	42 ± 12	6.2 ± 1.4	40 ± 12	4.8 ± 1.2
500	68 ± 20	7.8 ± 1.6	52 ± 20	6.8 ± 1.6

Note. Km and $V_{\max}$ derived from Lineweaver-Burk plots using regular regression analysis. (Samples from human cumulus of Patient No. 1, 7, and 17 in Table I).

DISCUSSION

The relationship between COH and ovarian carcinogenesis is controversial (12,13). Some investigators demonstrated the relationship between COH and the increased risk of ovarian tumors (12,14). However, there was no logic method or model to evaluate the ovarian status during COH. NAT is related with carcinogenesis (15). NAT activity also plays a major role in the detoxification of exogenous amine (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 organs of COH and oocyte retrievals, it is reasonable to speculate that cumulus cells may be involved in the acetylation of arylamine carcinogens.

NAT has been shown to exist 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 (6), liver (16), breast (20), small intestine (21), colon (2), lung (22), kidney (23), bladder (3), and prostate (23). However, there are no available reports about the acetylation of cumulus cell toward a carcinogen (2-AF) and a noncarcinogen (PABA). In this series, we established the ovarian NAT database after COH. We observed the higher percentage of rapid acetylators established in the cumulus during COH.

Two genetic classifications of arylamine NAT activity have been defined as monomorphic and polymorphic. Human and several species of animals show a polymorphism in NAT activity in their tissues (24). Thus, there are distinct groups of rapid and slow acetylators. Three phenotypes associated with the polymorphic pattern are rapid, intermediate, and slow

acetylators (25). Three variations are due to differences at the molecular level (25). Rapid and slow acetylations have been demonstrated as a predisposing factor for the sensitivity of individuals to the toxicity during exposure to many arylamines (26). It has been reported that rapid acetylators are related with a higher risk of colorectal cancer (27) and slow acetylators are 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.

There are at least two arylamine NATs for rabbits (28), hamsters (29), and humans (1), whereas three NATs have been found in mice (30). NAT1 and NAT2 have shown differences in substrate specificity. In mice, NAT1 did not catalyze the acetylation of PABA, but had activity for 2-AF. Mouse NAT2 catalyzed acetylation of both PABA and 2-AF (31). In contrast, human NAT1 acetylated both PABA and 2-AF, but human NAT2 acetylated 2-AF but not PABA (1). Both enzymes showed significant activity with carcinogenic arylamines such as 2-AF.

In this series, we observed that all cumulus samples had detectable levels of NAT activity. The two to four-fold higher Km value of rapid activity tissues compared to that of intermediate or low activity tissues suggested the possibility of multiple NAT isoforms in human cumulus cells. Higher Km/ $V_{\max}$ values suggest a second NAT isoform with different kinetic properties, because the cumulus cell cytosols were derived from whole homogenates. Thus, the data represent a mixture of tissue homogenates consisting of different cell types and multiple NAT isozymes. The heterogeneity was compatible with the determined kinetic constants. However, the role of arylamine NAT activity in the cumulus cells remains unknown, although

its usefulness in metabolism of endogenous amines is obvious.

In conclusion, this study provided the preliminary database about NAT activity in human cumulus cells. Human cumulus could acetylate arylamine carcinogen (2-AF) and noncarcinogen drug (PABA), which could lead to the development of a cumulus model for monitoring the effects of pollution on the gamete during COH. Our results support the multiple NAT isoforms in human cumulus cells. However, the real role of NAT activity upon the ovarian status during COH merits further investigation. Furthermore, the influences of patients' age, infertile causes, and COH protocol upon the NAT activity deserves further surveys. Also, whether the multiple COH treatment had the cumulative effect of NAT remains to be determined.

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