N-Acetyltransferase Expression and Metabolic Activation of the Food-derived Heterocyclic Amines in the Human Mammary Gland

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Abstract

The heterocyclic amines (HCAs) found in cooked meat are procarcinogens that are metabolically activated by N-hydroxylation followed by O-acetylation by the N-acetyltransferases NAT1 and NAT2. Despite the importance of metabolic activation in HCA carcinogenicity and the finding that several HCAs are rodent mammary gland carcinogens, nothing was known about O-acetylation activity in the human mammary gland. The current study examines the expression and catalytic activity of NAT toward the N-hydroxy-HCAs 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-f]pyridine (N-hydroxy-PhIP) and 2-hydroxyamino-3-methylimidazo[4,5-f]quinoline (N-hydroxy-IQ) in the human mammary gland. Mammary gland cytosol from 10 women and lysates from a first passage primary culture of human mammary epithelial cells metabolically activated 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-f]pyridine and 2-hydroxyamino-3-methylimidazo[4,5-f]quinoline by NAT-mediated O-acetyltransferase, as measured by the acetyl-CoA-enhanced binding of the N-hydroxylamines to calf thymus DNA in vitro. N-acetylation of p-aminosalicylic and aminophenanthridine, an activity specific to NAT1, but not N-acetylation of sulfamethazine, an activity specific to NAT2, was detected in the mammary gland cytosol and human mammary epithelial cell lysates. Immunohistochemical analysis of human mammary gland sections showed positive staining for NAT1 protein in the epithelial cells lining the mammary gland ducts. Reverse transcription-PCR analysis showed that mRNA transcripts for both NAT1 and NAT2 were present in human mammary gland; however, no NAT2 catalytic activity was detectable. Our data demonstrate for the first time that the human mammary gland is catalytically active toward the metabolic activation of HCA food mutagens, and that this activity is most likely contributed by NAT1 expressed in the ductular epithelial cells of the mammary gland.

Introduction

The etiology of human breast cancer is largely unknown; however, environmental factors, especially diet, appear to influence this disease (1). Humans are exposed to highly mutagenic and carcinogenic HCAs from the consumption of cooked meats (2). The finding that several HCAs, including PhIP and the quinolines IQ and 2-amino-3,4-dimethylimidazo[4,5-f]quinoline, are mammary gland carcinogens in rats raises the possibility that these compounds may play a role in human mammary gland cancer (reviewed in Ref. 3; Ref. 4). The HCAs, like many chemical carcinogens, require metabolic activation for DNA adduct formation and the initiation of carcinogenesis (3, 5). The first step in the metabolic activation of these compounds occurs by cytochrome P450-catalyzed N-hydroxylation (5). The resulting N-hydroxy derivatives are generally poorly reactive with DNA but can be further metabolized by N-acetyltransferase-mediated O-acetylation to esters that readily form DNA adducts (5–7). In humans, two N-acetyltransferases, designated NAT1 and NAT2, catalyze N- and O-acetylation of various arylamines, including the O-acetylation of N-hydroxy-HCAs (8–10). NAT2, and most recently NAT1, have been shown to be polymorphic enzymes that segregate individuals into rapid and slow acetylator phenotypes (10, 11). Genetic polymorphisms in these enzymes influence the balance of metabolic activation and detoxification of environmental arylamine carcinogens and may modulate the risk of certain human cancers such as those of the colorectum and bladder (12–15). Notably, recent studies have indicated that NAT1 activity in the human colon and bladder is an important determinant of cancer risk and in situ metabolic activation of arylamines (12–14). In light of these findings, it is possible that the susceptibility of the human mammary gland to arylamine carcinogens, such as the dietary HCAs, may also be influenced by intramammary NAT1 or NAT2 activity. However, nothing is known about the NAT enzymes in the human mammary gland. In this study, we examine the expression and activity of NAT1 and NAT2 in the human mammary gland with the objective of determining if the human mammary gland has the capacity to carry out the metabolic activation of the N-hydroxy-HCAs.

Materials and Methods

Chemicals and Antibodies. N-Hydroxy-IQ and N-hydroxy-PhIP were synthesized as described previously (6). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO). N-Acetylated SMZ and a polyclonal antibody raised in rabbit against a recombinant human NAT1 protein expressed in Escherichia coli were provided by Dr. Denis Grant (Hospital for Sick Children, Toronto, Ontario, Canada).

Animals. Female Sprague-Dawley rats were obtained from NIH Animal Supply (Frederick, MD) at 60–70 days of age and maintained on NIH Lab Chow and water ad libitum. Mammary gland and liver cytosol were prepared as described below for human samples.

Preparation of Human Mammary Gland Cytosol. Snap-frozen human mammary gland samples from patients aged 15–69 years old, undergoing reduction mammoplasty, were obtained from The Cooperative Human Tissue Network and stored at −80°C until use. Cytosolic fractions were isolated by differential ultracentrifugation (16). Five to 10 g of tissue was homogenized with a Polytron in 10 ml of TEDK buffer (10 mM triethanolamine-HCl, 1 mM EDTA, 1 mM DTT, and 50 mM KCl, pH 7.0) containing 0.02 mM leupeptin, 0.1 mM PMSF, 0.05 mM butylated hydroxytoluene, and 1 μM pepstatin A. The protein concentration of the cytosols was determined colorimetrically by the BCA protein assay (Pierce, Rockford, IL). Due to the lability of the NAT enzyme, all NAT enzyme assays were run immediately following cytosol isolation.

Cell Culture and Preparation of Cell Lysates. A primary culture of HMECs, derived from a 50-year-old healthy woman who had undergone reduction mammoplasty, was purchased from Clonetics (San Diego, CA). At passage number 9 or 10, cells from nine confluent T75 flasks were trypsinized, and three cell pellets, constituting a pool of three T75 flasks each, were collected by centrifugation at 4°C. Subsequently, the cell pellets were washed
and resuspended in 1 ml ice-cold TEDK buffer. The cells were sonicated, and the cell debris was removed by brief centrifugation. The protein concentration was determined, and the lysate was used immediately for the NAT enzyme assays described below.

**N-Acetyltransferase Assay.** SMZ N-acetylation, a measure of NAT2 activity, and PAS N-acetylation, a measure of NAT1 activity, were determined spectrophotometrically essentially as described previously (17). The incubations contained 0.25 mM AcCoA, 20 µl of an AcCoA regenerating system (5.4 mg acetyl-CoA-carnitine and 1 unit carnitine acetyltransferase), 200 µM substrate (SMZ or PAS), and 50 µl of mammary gland cytosol or epithelial cell lysate (0.25–0.5 mg protein) in a total volume of 100 µl. Concomitantly, control incubations were run that did not contain AcCoA. Following a 20-minute incubation at 37°C, the reactions were stopped by the addition of 50 µl of 20% trichloroacetic acid and centrifugation. One ml of 2.5% dimethylaminobenzaldehyde in acetonitrile was added to the supernatants. After 10 min, the absorbance at 450 nm was measured. The level of acetylation, measured as a decrease in the absorbance at 450 nm, was determined using a standard curve for each substrate. The assays were run under conditions of linearity for incubation time and cytosolic protein concentration. The limit of detection of product was 0.1 nmol/min/mg protein of acetylated product in the total incubation mixtures.

SMZ N-acetylation in human mammary gland cytosol was also measured by HPLC. Incubations were carried out as described above and terminated by the addition of 10 µl of 15% perchloric acid and centrifugation. Fifty µl of supernatant were analyzed by HPLC, essentially as described previously (18). HPLC was carried out with a Beckman Ultrasphere reversed-phase C18 ODS column (5 µm; 4.6 x 150 mm) using a Gilson model 715 system equipped with a Groton photodiode array detector. The solvent condition was initially 10% acetonitrile in 20 mM sodium perchlorate buffer (pH 2.5) and followed a linear gradient to 20% acetonitrile over an 8-min period. The flow rate was 1.5 ml throughout the run. The retention time of the N-acetylated SMZ was confirmed using synthetic standards. A positive control for SMZ N-acetylation activity was run using rat and mouse hepatic cytosolic fractions. The limit of detection was 0.05 nmol/min/mg protein.

**O-Acetyltransferase Assay.** NAT-mediated O-acetylation of N-hydroxy-PhIP and N-hydroxy-IQ was assayed in mammary gland cytosol and in HMEC lysates by the AcCoA-enhanced binding of the hydroxylamines to calf thymus DNA, as described previously (7). DNA was isolated by phenol extraction, and IQ- and PhIP-DNA adduct levels were quantitated by the 32P-postlabeling method, as described previously (6). DNA adduct values were expressed as RAL × 107.

**RT-PCR and RFLP Analysis of NAT1 and NAT2.** The RT-PCR analysis was carried out using the NAT1 and NAT2-specific primers reported previously by Kloth et al. (19). Total RNA was isolated from frozen human mammary glands, reverse transcribed and PCR amplified as described previously (20). The authenticity of the amplified alleles was confirmed by RFLP analysis using HincII (which digests NAT2 to produce 659- and 248-bp fragments) and HincIII (which digests NAT1 to produce 786- and 75-bp fragments) (19).

**Immunohistochemical Localization of NAT1 in the Human Mammary Gland.** Normal human mammary gland samples, fixed in 10% formalin and embedded in paraffin, were obtained from the Cooperative Human Tissue Network and cut into 5-µm sections. Following deparaffinization, the sections were blocked with a dilution of goat serum for 1 h and subsequently incubated overnight with a polyclonal antibody prepared against recombinant human NAT1. The NAT1 protein was localized using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). To exclude the possibility of nonspecific binding of the secondary antibody to human mammary gland sections, control sections were run that lacked the primary antibody. In addition, other control sections were run using rabbit serum (1:50 dilution) instead of the NAT1-specific antibody. All the slides were subsequently counterstained with hematoxylin.

**Results**

Mammary gland cytosol from 10 women carried out the NAT-catalyzed AcCoA-mediated binding of N-hydroxy-PhIP to calf thymus DNA (Fig. 1A). The binding of N-hydroxy-PhIP to calf thymus DNA was linear, with substrate up to 100 µM per incubation (Fig. 1B). The adduc-
Table 1  *N*-Acetyltransferase activity in human mammary gland cytosol and in human mammary epithelial cells

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Mammary gland cytosol</th>
<th>HMEC lysates</th>
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<tbody>
<tr>
<td></td>
<td>n</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>N-OH-IQ</td>
<td>10</td>
<td>2.34 ± 1.56</td>
</tr>
<tr>
<td>N-OH-PhIP</td>
<td>10</td>
<td>0.94 ± 0.50</td>
</tr>
<tr>
<td>PAS</td>
<td>8</td>
<td>395 ± 125</td>
</tr>
<tr>
<td>SMZ</td>
<td>8</td>
<td>ND</td>
</tr>
</tbody>
</table>

aN Human mammary gland cytosol was prepared as described in “Materials and Methods.”

bA primary culture of human mammary epithelial cells was lysed by sonication, and the cell lysates were used.

cNumber of individual human mammary gland cytosol samples that were assayed in duplicate.

dMean of three to four separate experiments.

eRelative adduct level (RAL) × 10⁷ nucleotides/mg of cytosolic protein/20-min incubation.

fPmol of N-acetylated product formed/minute/mg of cytosolic protein.

ND, not detected.

Detected with any of the human or rat mammary gland samples or in HMEC lysates (Table 1). SMZ N-acetylation was, however, found in rat liver cytosol (167.33 ± 73.13 pmol/min/mg protein, mean ± SD, n = 4). In addition to the standard colorimetric assay, N-acetyl-SMZ could not be detected in incubations with human mammary gland cytosol or HMEC lysate, using an HPLC method. Pentachlorophenol (250 μM), an inhibitor of NAT activity, inhibited PAS N-acetylation and N-hydroxy-PhIP O-acetylation reactions by an average of 60% in both human mammary gland cytosolic incubations and in HMEC cell lysate incubations (data not shown).

Immunohistochemical analysis of human mammary gland samples showed positive staining using an NAT1-specific antibody (Fig. 2). The staining was limited to the ductular epithelial cells of the mammary gland. Notably, no staining was observed in control slides where the primary antibody was omitted (Fig. 2, B and F) or in samples incubated with rabbit serum (Fig. 2D).

RNA was isolated from frozen human mammary gland samples and reverse transcribed using NAT1- and NAT2-specific primers, as described previously (19). When the total RNA from 12 individual women was isolated and analyzed, all showed the presence of NAT1 and NAT2 transcripts (Fig. 3A). The authenticity of the products was confirmed by RFLP analysis using HindII, which digests the NAT2 product (907 bp) into two fragments (659 and 248 bp), and HindIII, which cleaves the NAT1 product (861 bp) into a 786-bp fragment and 75-bp fragment (Fig. 3B).

Discussion

The metabolic activation of the HCAs at particular tissue sites may be one important determinant of the target organ specificity for HCA.
NAT EXPRESSION AND FOOD-DERIVED HETEROCYCLIC AMINES

Fig. 3. Expression of NAT1 and NAT2 in human mammary gland as determined by RT-PCR analysis. A, RT-PCR analysis of human mammary gland RNA from 10 women using NAT1 and NAT2-specific primers. B, RFLP patterns of NAT1 (Lanes 1–3) and NAT2 (Lanes 4–6) from the PCR amplification of mammary gland RNA was digested with HindII (Lanes 2 and 5) or HindIII (Lanes 3 and 6), or without enzymes (Lanes 1 and 4). Arrows, NAT1 and NAT2 PCR products and restriction fragment sizes. RFLP is shown for one representative individual. L, ladder.

carcinogenesis. With regard to the mammary gland, studies in rats have suggested that the in situ O-acetylation capacity of the rat mammary gland may be associated with the susceptibility of the gland to PhIP-induced carcinogenesis (21). With the present study, we demonstrate, for the first time, that NAT1 is expressed in the human mammary gland ductular epithelial cells and that it is catalytically active toward the N-acetylation of PAS and the O-acetylation of N-hydroxyamines. Human mammary gland cytosols catalyzed the AcCoA-dependent O-acetyltransferase-mediated binding of N-hydroxy-PhIP and N-hydroxy-IQ to DNA in vitro. This activity was also observed in lysates of a primary culture of HMECs. Comparison of the O-acetylation activity observed in human mammary gland cytosol with that reported previously for rat mammary gland cytosol (21) indicates that O-acetylation of N-hydroxy-PhIP and N-hydroxy-IQ is exceptionally high in the rat, despite the similar activity of PAS N-acetylation (reported herein). This finding suggests, in part, that the N-hydroxy-HCA substrate specificity is different between the rat and the human mammary gland NATs. Although the limited in vitro data suggest that the human may be less susceptible than the rat to HCA-induced mammary carcinogenesis, it may be hasty to speculate on the relative susceptibility of the human mammary gland to the HCAs. Nonetheless, the finding that human mammary gland NAT is catalytically active toward N-hydroxy-HCA is consistent with the notion that the human mammary gland may be susceptible to the carcinogenic effects of these compounds.

In humans, two N-acetyltransferases, NAT1 and NAT2, can O-acetylate the N-hydroxyamine derivatives of the HCAs (8–9). The results from the studies shown here suggest that O-acetylation of N-hydroxy-PhIP and N-hydroxy-IQ in the human mammary gland is largely carried out by NAT1. This conclusion is supported by the finding that SMZ N-acetylation, an NAT2 activity, was not detectable in mammary gland cytosols or HMEC cell lysate, whereas PAS N-acetylation, an NAT1 activity, was clearly observed in both the cytosols and cell lysates. The results from RT-PCR, although not quantitative, indicate that NAT2 as well as NAT1 mRNA is expressed, and thus we cannot rule out the possibility of a very low level of NAT2 activity in the human mammary gland. Nevertheless, the conclusion that NAT1 is the predominant NAT responsible for the N-acetyltransferase activity in the human mammary gland is consistent with the finding that other extrahepatic tissues, such as colon and bladder epithelium, show NAT1 activity but lack detectable NAT2 activity (14, 22). In addition, the results from our immunohistochemical studies of the human mammary gland using an NAT1-specific antibody further demonstrate the expression of NAT1 protein in the epithelium lining the ducts of the human mammary gland.

Recent studies have shown that NAT1, previously referred to as a monomorphic enzyme, is polymorphic owing to allelic variances that alter tissue levels of the enzyme and hence NAT1 activity (11–14). In the human bladder, a specific NAT1 genetic polymorphism (NAT1*10 allele), which increases NAT1 expression in the bladder, is associated with higher carcinogen-DNA adduct levels (14). Furthermore, studies in the human colon showed that NAT1 genetic polymorphisms that increase NAT1 activity also increase the risk of colorectal cancer (12, 13). Thus, the evidence to date suggests that NAT1 genetic polymorphisms that cause variations in in situ metabolic activation may play an important role in target organ susceptibility. Notably, our results show that NAT1 is expressed in the ductal epithelial cells, which are considered to be the cells associated with mammary gland cancer (23). Thus, metabolic activation of the N-hydroxylamines by O-acetylation is likely to occur at the critical site for the initiation of mammary gland cancer by the HCAs. In addition, the variation in N-hydroxy-PhIP O-acetylation observed in the mammary gland samples raises the possibility that the NAT1 genotype, and hence susceptibility to HCA carcinogenesis, may be different among the women we examined.

Several epidemiological studies have failed to show an association between NAT acetylation phenotype and the incidence of breast cancer in women (24, 25). However, these studies measured N-acetylated SMZ in blood samples from women given SMZ and, therefore, assessed the NAT2 rather than the NAT1 acetylator phenotype. In light of previous studies supporting the association between the NAT1 genotype and colorectal cancer and our current study showing that NAT1 is catalytically active and expressed in the human mammary gland, additional studies are needed to determine if the NAT1 genotype is a risk factor for human mammary gland cancer. In addition, to better assess the role of dietary HCAs in human breast cancer, the NAT1 genotype and human mammary gland cancer incidence among women who regularly eat well-done cooked meats is warranted.

Acknowledgments

We thank Dr. Denis G. Grant (Hospital for Sick Children, Toronto, Ontario, Canada) for providing the polyclonal anti-human NAT1 antibody and technical advice. Dr. Herman A. J. Schut (Medical College of Ohio, Toledo, Ohio) for the 32P-postlabeling analysis, and Dr. Snorri S. Thorgerisson (National Cancer Institute, Bethesda, Maryland) for support and helpful discussions.

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