**N-Acetyltransferase expression and DNA binding of N-hydroxyheterocyclic amines in human prostate epithelium**

Ching Y.Wang¹, Maria Debiec-Rychter¹, Herman A.J.Schut², Philo Morse, Richard F.Jones, Corlis Archer, Charles M.King³ and Gabriel P.Haas

Department of Urology, SUNY Health Science Center and VA Medical Center, Syracuse, NY 13210, USA, ¹Department of Oncology, Medical Academy of Lodz, Lodz, Poland, ²Department of Pathology, Medical College of Ohio, Toledo, OH 43614 and ³5009 Kelsie Court, Florence, OR 97439, USA

Intact prostate epithelial cells prepared from benign prostatic hypertrophy tissues from two patients were incubated for 2 h with N-hydroxy derivatives of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (N-OH-PhIP) or 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (N-OH-MeIQx). ³²P-post-labeling analysis detected PhIP and MeIQx adducts in the DNA of these cells but not in the untreated control. Adduct levels were ~100 times greater in N-OH-PhIP- than in N-OH-MeIQx-treated cells. Repair synthesis of DNA was observed in cells, prepared from two additional patients, treated for 24 h with these carcinogens and was greater for N-OH-PhIP than for N-OH-MeIQx. PhIP, MeIQx and their nitro derivatives did not produce repair synthesis of DNA in this system. The difference in the activity of N-OH-PhIP and N-OH-MeIQx may be due to their stability, since N-OH-MeIQx decomposed rapidly in neutral solution. Transcripts of NAT1 and NAT2 were detected by an in situ hybridization method in prostate epithelial cells, but were absent from stromal tissues. These results suggest that PhIP may be a potential carcinogen for human prostate, since cooked meats, which contain this heterocyclic amine, have been associated with human prostate cancer.

**Introduction**

The etiology of prostate cancer is not well understood, although evidence from epidemiological studies suggests that exogenous agents may contribute to the development of this cancer. Food components, such as meat and fish, have been associated with increases in prostate cancer incidence in different ethnic immigrants in the USA as compared with those living in their native countries (1,2). Cooked meat and fried fish contain components, such as meat and fish, have been associated with increases in prostate cancer incidence in different ethnic immigrants in the USA as compared with those living in their native countries (1,2). Cooked meat and fried fish contain carcinogens and was greater for N-OH-PhIP than for N-OH-MeIQx. PhIP, MeIQx and their nitro derivatives did not produce repair synthesis of DNA in this system. The difference in the activity of N-OH-PhIP and N-OH-MeIQx may be due to their stability, since N-OH-MeIQx decomposed rapidly in neutral solution. Transcripts of NAT1 and NAT2 were detected by an in situ hybridization method in prostate epithelial cells, but were absent from stromal tissues. These results suggest that PhIP may be a potential carcinogen for human prostate, since cooked meats, which contain this heterocyclic amine, have been associated with human prostate cancer.

**Materials and methods**

**Chemicals**

PhIP and MeIQx were purchased from Toronto Research Chemical Co. (North York, Ontario, Canada). Nitro-PhIP was synthesized using a slight modification of a published method (13). Nitro-PhIP was extracted into chloroform after the reaction of PhIP with NaNO₂. The chloroform extract was immediately washed with 1 M sodium bicarbonate, dried over anhydrous sodium sulfate and then applied to a silica gel column. Nitro-PhIP was eluted from the column with 0.4% MeOH in chloroform. The yield of nitro-PhIP was 49%. UV spectroscopy showed absorption maxima in methanol at 245 and 354 nm (3). Addition of this nitro compound with hydrazine yielded N-OH-PhIP (13). N-OH-MeIQx was prepared from MeIQx as described (14).

Although generally not regarded as a risk factor for prostate cancer, the mortality rate of this cancer is higher in smokers than in non-smokers (5).

Most aromatic amines and HCAs, after undergoing bioactivation, can react with genomic DNA to form covalent adducts. Although DNA modification may not alone be sufficient for tumor formation, the formation and persistence of DNA adducts have been correlated with the cytotoxic, mutagenic and carcinogenic effects of these agents (6). Following N-oxidation by cytochrome CYP1A2, aromatic amines and HCAs can be activated by N-acetyltransferases (NATs) to reactive N-acetoxyamines (7). Target tissues of carcinogenesis produced by aromatic amines and HCAs in various species invariably contain NAT (8). The two NAT isozymes, NAT1 and NAT2, differ significantly in their intrinsic organ-specific expression, stabilities and substrate specificities and heritable sequence polymorphisms account for differences that permit segregation of individuals into rapid and slow acetylator phenotypes. The possible significance of NATs in human cancer is evident in the observation that both phenotypes of acetylation capacity, rapid and slow, have been reported to be susceptibility factors for colon and bladder cancers (9–11), which may be caused by aromatic amines or HCAs.

PhIP and 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) are present in cooked meat (3) and PhIP is also present in cigarette smoke (4). PhIP forms DNA adducts and carcinomas in the prostate of rats ingesting this compound and it is suspected to cause prostate cancer in men (12). Nevertheless, little is known about the capability of human prostate to activate these carcinogens. In the present study, we examined human prostate epithelium for the expression of NAT genes and their capacity to activate N-hydroxy-2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (N-OH-PhIP) and N-hydroxy-2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (N-OH-MeIQx), as reflected by increases in DNA adduct formation and unscheduled DNA synthesis (UDS).
Concentration of methanol was then linearly increased to 50% over 20 min. The elution was maintained at 0.1% dimethylamine/50% methanol for an additional 3 min and the concentration of methanol was then decreased linearly to 20% over the next 4 min. The elution times were 18.1, 19.1 and 26.3 min for MeIQx, N-OH-MeIQx and nitro-MeIQx, respectively. The purities of N-OH-PhIP and N-OH-MeIQx were >90% as judged by the elution profiles. Nitro and amine derivatives were the major impurities.

**Stability of N-OH-PhIP and N-OH-MeIQx**

Phosphate-buffered saline (PBS) solutions (pH 7.0) containing 1 μM N-OH-PhIP or N-OH-MeIQx were incubated at 37°C. At various intervals, the solutions were subjected to analysis using HPLC as described above and the half-lives of these two compounds in the buffer solution were determined.

**Prostate tissues**

Prostate tissues (2–4 g) were obtained from six Caucasian patients, aged 65–70 years, who had undergone transurethral prostatectomy for benign prostate hypertrophy (BPH). Samples from two patients each were used for experiments on UDS detection, DNA adduct analysis and in situ hybridization (ISH), described below.

**Preparation of prostate epithelium**

The procedure of Rheim et al. (15) with modification was used. Prostate tissue (2–4 g) was placed in a Petri dish and cut into ~1–5 mm³ fragments. The fragments were rinsed twice with PBS to remove fine debris and then suspended in 50 ml Earl’s minimal essential medium (EMEM) containing 10 mM HEPES, 5% newborn bovine serum and 20 000 U collagenase (Gibco, Grand Island, NY). The suspension was incubated with continuous shaking at 37°C for 2 h, pipetted vigorously and then centrifuged. The cell pellet was suspended in PBS, allowed to settle for 15 min and the precipitate was collected. This procedure was repeated twice and the final cell pellet was suspended and vigorously pipetted in serum-free EMEM to yield prostate epithelial cells. One drop of the cell suspension was placed on a coated histological slide, air dried, fixed in methanol and immunohistochemically stained with CYT-351 mAb (Cytogen, Princeton, NJ), which is specific for prostate-specific membrane antigen, using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA). CYT-351 mAb was diluted 500 times before use. The staining procedure was according to the manual supplied with the kit. Normal mouse serum was used as a control. The slide was examined under a light microscope.

**UDS**

Freshly prepared prostate epithelial cells (~5×10⁶ cells) were suspended in 1 ml of serum-free EMEM medium containing 0.5 μCi[³H]thymidine, 10 μmol hydroxyurea and the test compound in 0.01 ml of dimethylsulfoxide. After incubation at 37°C for 24 h in a humidified atmosphere of 5% CO₂, the cell suspension was centrifuged and the pelleted cells washed with PBS, treated with 1% sodium citrate and fixed in MeOH/acidic (3:1 v/v) solution for 15 min. Following centrifugation, pelleted cells were suspended in a small amount of the fixative, then dropped onto an ice-cold slide. The slide was allowed to air dry, washed with deionized water and coated with autoradiographic emulsion. After storage in the dark for 2 weeks, slides were developed and scored for grain counts/nucleus. HCV-29 (16), a human urothelial cell line previously shown to be responsive to UDS by hydroxylamines, was used as a positive control (16).

As reported previously, HCV-29 cells were allowed to attach on a coverslip, then treated with [³H]thymidine, hydroxyurea and carcinogens (16). The concentrations of carcinogens used for the experiment shown in Table I were selected from a preliminary experiment.

**DNA adduct formation**

Prostate epithelium (~2×10⁷ cells), prepared as described above from two BPH patients, were suspended in 1 ml of serum-free DMEM containing the desired amount of N-OH-PhIP or N-OH-MeIQx (selected from the results of the UDS experiments) and incubated in a humidified atmosphere of 5% CO₂ at 37°C for 2 h. The cells were then washed three times with PBS. A suspension of HCV-29 cells was treated in a similar manner for 2 h. DNA was isolated from pelleted cells, using a direct salting-out procedure as described before (17). PhIP–DNA adducts were isolated and quantified by ³²P-post-labeling as described previously (17,18), using the intensification version of the assay (19). Adduct levels (sum of individual adducts) were expressed as relative adduct labeling (RAL) values after correction for intensification factors (17). dG-C⁸–PhIP and dG-C⁸–MeIQx were identified by co-migration with reference compounds as described previously (18).

**Detection of NAT transcripts by ISH**

Fresh prostate specimens from two BPH patients were fixed in 4% paraformaldehyde in PBS, pH 7.4, for 2 h at 4°C. Immediate tissue processing and short fixation times were essential for successful detection of transcripts by ISH. The prostate specimens were embedded in paraffin and 6–8 mm sections were mounted on Vectabond-coated slides (Vector Laboratories) under RNase-free conditions. The slides were dried for 1 h at 55°C and stored at room temperature until use. The hybridization probes consisted of a mixture of six 24–44 base antisense oligonucleotides (Midland Certified Reagent Co., Midland, TX), complementary either to human NAT1 or NAT2 mRNAs in regions chosen to maximize sequence differences between the two transcripts (GenBank accession nos: NAT1, D90041; NAT2, D90042). Tₘ values of the probes were within 1°C for their respective target transcripts as determined by nearest-neighbor analysis (Oligo 5.0 software; National Biosciences, Hamel, MN). The oligo probes were digoxigenin end-labeled with terminal transferase (Boehringer Mannheim Biochemicals, Indianapolis, IN) as described previously (20). The human NAT1 antisense probes were as follows (5’→3’): TCTT TTCCT TCCGT CAAAC GGAAG ACA; GAAGA AA TTC TTCA

**Table I. Induction of UDS by N-OH-PhIP and N-OH-MeIQx**

<table>
<thead>
<tr>
<th>Cells</th>
<th>Compound</th>
<th>Concentration (M)</th>
<th>UDS (grains/nucleus, means ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCV-29</td>
<td>Control</td>
<td>10⁻⁶</td>
<td>11.3 ± 4.9</td>
</tr>
<tr>
<td>N-OH-PhIP</td>
<td>10⁻⁵</td>
<td>&gt;100</td>
<td></td>
</tr>
<tr>
<td>10⁻⁷</td>
<td>71.1 ± 14.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10⁻⁸</td>
<td>13.0 ± 9.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-OH-MeIQx</td>
<td>10⁻⁴</td>
<td>80.5 ± 5.8</td>
<td></td>
</tr>
<tr>
<td>10⁻⁵</td>
<td>39.0 ± 7.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prostate</td>
<td>Control</td>
<td>10⁻⁵</td>
<td>1.7 ± 3.7</td>
</tr>
<tr>
<td>N-OH-PhIP</td>
<td>10⁻⁶</td>
<td>&gt;100</td>
<td></td>
</tr>
<tr>
<td>10⁻⁷</td>
<td>39.4 ± 17.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10⁻⁸</td>
<td>2.6 ± 5.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-OH-MeIQx</td>
<td>10⁻⁴</td>
<td>15.7 ± 7.3</td>
<td></td>
</tr>
<tr>
<td>10⁻⁵</td>
<td>10.0 ± 5.6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Values are from the evaluation of 25–30 nuclei.

**Fig. 1. Detection of DNA adducts under intensification conditions in human prostate epithelium with ³²P-post-labeling following the treatment with N-OH-PhIP (A) or N-OH-MeIQx (B).** After correction for intensification factors, spot 1 is the major adducts of PhIP and MeIQx, identified as dG-C⁸–PhIP (A) and dG-C⁸–MeIQx (B), respectively (18). Minor PhIP– DNA adduct 4, which was not detected under standard ATP saturation labeling conditions (17), represented 6.6–15.7% of the total intensified adducts.

**Table II. Formation of DNA adducts by N-OH-PhIP and N-OH-MeIQx**

<table>
<thead>
<tr>
<th>Cells</th>
<th>HCA</th>
<th>Concentration (M)</th>
<th>Total adducts (RAL×10⁴)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCV-29</td>
<td>N-OH-PhIP</td>
<td>10⁻⁵</td>
<td>63.08</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10⁻⁶</td>
<td>6.43</td>
</tr>
<tr>
<td></td>
<td>N-OH-MeIQx</td>
<td>10⁻⁵</td>
<td>0.49</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10⁻⁶</td>
<td>0.11</td>
</tr>
<tr>
<td>BPH1</td>
<td>N-OH-PhIP</td>
<td>10⁻⁵</td>
<td>13.46</td>
</tr>
<tr>
<td></td>
<td>N-OH-MeIQx</td>
<td>10⁻⁵</td>
<td>0.06</td>
</tr>
<tr>
<td>BPH2</td>
<td>N-OH-PhIP</td>
<td>10⁻⁵</td>
<td>21.63</td>
</tr>
<tr>
<td></td>
<td>N-OH-MeIQx</td>
<td>10⁻⁵</td>
<td>0.64</td>
</tr>
</tbody>
</table>
TTTG GAATG TACTG TGAGG TT; GAGTA AAGGA GTAGA TTGGG TT; GAGTA AAGGA GTAGA TTTTT
CGGTA TTTGC TGTCT TCT; GGAAC AAAA TGA TTT ACTAG TAAAC
ACAGA TGA TG GAGA T; ACTTT TTCTA TTTCT TCCTC ACTCAG
AGTCT TGAAC TCTA T T; AAAAA A TCTA TCACC A TGTT TGGGC
ACAAG C. The human NAT2 antisense probes were (5'-3'):
GGAT CTCCT TCTTC TGTCA AGCAG AAAA TGC; TGAGA A TAA GA TCTTT
GTTTG TAATA TACTG TCTTC TCT; GGGTA AATAA GTATA TTTTT
TGTTG TTTCT TCTTT GGC; ACAAA A TGA TGTGGT TA TAA A TGAAG
AGTGT GAGGA C; AACCT CTTCC TCAGTG AG; AGTTT TA AAC
TCGAC C; GGGAT CCA TC ACCAG GTTTG GGCA. As a negative control,
a mixture of sense oligodeoxynucleotides for each corresponding antisense probe was used. The ISH procedure was essentially as described previously (20). Briefly, after deparaffinization and rehydration, the slides were microwaved in RNase-free 10 mM citrate, pH 6.0, for 20 min, treated with 5 µg/ml proteinase K for 15 min at 37°C. Hybridization was performed at 42°C for 16 h (20). Control hybridizations were carried out simultaneously using sense probes. The post-hybridization washes included two changes of 50% formamide, 40 mM NaCl, 40 mM sodium citrate for 30 min each at 42°C, followed by two changes of 40 mM NaCl, 40 mM sodium citrate (pH 7.4) for 30 min each at room temperature. For detection of hybridized probe, the sections were incubated with alkaline phosphatase-conjugated antidigoxigenin antibody (dilution 1:200; Boehringer Mannheim) and developed with the one-step nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate (Pierce Chemical Co., Rockford, IL).

Results

Induction of UDS

Because fibromuscular tissue is a major component of BPH, fibrous debris could not be completely removed from the cell preparation with the procedure used. However, ~90% of the cells in the preparation stained positive for prostate-specific membrane antigen. As shown in Table I, both N-OH-PhIP and N-OH-MeIQx induced extensive UDS relative to untreated controls in these cells. N-OH-PhIP was ~100 times more active than N-OH-MeIQx for both the prostate cells and the HCV-29 human bladder cells. PhIP, nitro-PhIP, MeIQx and nitro-MeIQx, which are the major impurities in the N-hydroxy preparations, did not produce UDS (data not shown).

DNA adduct formation

Prostate epithelial cell cultures prepared as before from two individual patients were incubated with N-OH-PhIP or N-OH-MeIQx for 2 h at 37°C. The human bladder cell line HCV-29 was included as a positive control. All samples incubated with N-OH-PhIP or N-OH-MeIQx contained DNA adducts, which were detected by autoradiography of the thin-layer chromatography plates (Figure 1), then quantified by Cerenkov counting of the individual adduct spots taken from the plates. The numbers of adducted nucleotides, expressed as RAL values, are shown in Table II. With both N-OH-PhIP and N-OH-MeIQx as substrates, adduct formation by HCV-29 cells was related to substrate concentration; PhIP–DNA adduct levels were ~50–100 fold higher than MeIQx–DNA adduct levels. Similarly, adduct formation by BPH epithelial cells was much higher with N-OH-PhIP than with N-OH-MeIQx and variability between the two BPH samples was much higher with MeIQx–DNA adducts than with PhIP–DNA adducts (Table II). No adducts were detected in control cultures incubated without added HCAs.

Expression of NAT1 and NAT2 transcripts

Both, NAT1- and NAT2-specific mRNAs were detected in the glandular prostate epithelium, giving hybridization signals of relatively similar intensity for both probes (Figure 2). In contrast, the loose connective tissue and the capillary vessels of the stroma, which surround the glandular prostate, did not show any significant hybridization signal. As before (20), the hybridization signal seen under these conditions was cytoplasmic, as would be expected for transcript localization. Essentially no signal was detected in control sections hybridized with sense probes in any of these experiments.

Stability of N-OH-PhIP and N-OH-MeIQx

In PBS solution, pH 7.0, N-OH-PhIP was much more stable than N-OH-MeIQx. As determined by HPLC, the half-life of N-OH-PhIP was ~50 min, whereas it was <5 min for N-OH-MeIQx.

Discussion

BPH arises from the central and transitional zones of the prostate, where ~30% of prostate cancers occur (21). Because

Fig. 2. Detection of NAT transcripts in human prostate with ISH. (a) ISH with antisense NAT1 probes; (b) ISH with sense NAT1 probes; (c) ISH with antisense NAT2 probes; (d) ISH with sense NAT2 probes.
BPH samples are readily available from patients undergoing transurethral resection, they were used for the present study. The use of these cells can demonstrate a terminal metabolic activation of carcinogens to DNA-reactive products that result in DNA damage and adduct formation in these target cells. We have demonstrated that N-hydroxy derivatives of two of the most abundant HCAs in cooked foods, PhIP and MeIQx, can cause DNA damage and produce DNA adducts in human prostate epithelial cells in vitro. Consistent with previous studies, the major DNA products were dG-C8-PhIP and dG-C8-melQx (17,18). The presence in prostate cells of transcripts for NAT1 and NAT2, corroborated by the detection of NAT2 enzyme in prostate tissues (22) that can generate these adducts (23), supports the conclusion that these enzymes are likely to be responsible for the activation of these N-hydroxy metabolites in this organ. The observation that neither PhIP nor MelQx induced UDS in prostate epithelial cells suggests that oxidation by either the cytochrome or peroxidase systems may not be important in the terminal activation of these amines in these cells. Consistent with a previous report (24), we observed that N-OH-MelQx decomposed rapidly in neutral saline solution. The half-life of N-OH-MelQx was ~10 times shorter than that of N-OH-PhIP and may account for the observation that the former was ~50–100 times less active than the latter in inducing UDS and adduct formation (Tables I and II). The involvement of NATs can be further ascertained by using chemicals such as pentachlorophenol to inhibit these enzymes. However, we found that this compound was too toxic for use in human prostate epithelial cells (data not shown).

In vitro studies have demonstrated that HCAs can be activated by N-hydroxylation with CYP1A2 followed by O-esterification (7). The structures of at least two of the nucleotide adducts detected in the present study are consistent with C-8 adducts found in various tissues in animals fed with the parental amines and those from O-acetylation of N-OH-PhIP and N-OH-MelQx in vitro (23). The fact that targets of carcinogenesis for aromatic amines and HCAs in animals invariably express NATs demonstrates the probable importance of O-acetylation in the carcinogenesis of aromatic amines and HCAs (11).

PhIP and MelQx are abundant HCAs formed when meat is cooked at high temperatures. They produce tumors in several organs in the rat (25). Humans eating cooked meats excrete these compounds and their metabolites in the urine (26,27), thus demonstrating human exposures to these HCAs. Data presented herein have demonstrated the expression of NAT transcripts in prostate cells and the capability of these cells to activate N-hydroxy derivatives of these HCAs. HCAs, such as PhIP, are, therefore, potential carcinogens for human prostate, a conclusion consistent with the idea that cooked meat is a causal factor for prostate cancer.

Acknowledgements

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References

            levels of urinary 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx)
            (1997) Urinary excretion of unmetabolized and phase II conjugates
            of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine and 2-amino-3,8-
            dimethylimidazo[4,5-f]quinoxaline in humans: relationship to cytochrome

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