Bioactivation of the cooked food mutagen N-hydroxy-2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine by estrogen sulfotransferase in cultured human mammary epithelial cells

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Cooked food mutagens from fried meat and fish have recently been suggested to contribute to the etiology of breast cancer. Thus, the most prevalent of these compounds, i.e. 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine, or rather its more mutagenic N-hydroxy metabolite (N-OH-PhIP), forms DNA adducts in mammalian cells, including human mammary epithelial (HME) cells. The objective of this study was to determine the involvement of estrogen sulfotransferase (EST), the only sulfotransferase identified in HME cells, in the further bioactivation of N-OH-PhIP. These studies were done in vitro using human recombinant EST and in intact HME cells. Human recombinant EST increased the covalent binding of [3H]N-OH-PhIP to calf thymus DNA ~3.5-fold in the presence of the sulfotransferase co-substrate 3'-phosphoadenosine-5'-phosphosulfate at each N-OH-PhIP concentration (1, 10 and 100 µM) (n = 6, P < 0.001). In contrast, EST did not catalyze the DNA binding of two other cooked food mutagens, N-hydroxy-2-amino-3-methylimidazo[4,5-f]quinoline and N-hydroxy-2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline, which are mainly hepatocarcinogens. Cultured HME cells displayed high EST activity, which could be completely inhibited by 1 µM estrone. When the cells were incubated with [3H]N-OH-PhIP, binding to native DNA occurred at 60–240 pmol/mg DNA. This binding was inhibited to 55% of control by 1 µM estrone (P < 0.01, n = 8), suggesting that EST plays a significant role in carcinogen bioactivation in human breast tissue.

Breast cancer is the second leading cause of cancer-related death in American women (1), yet its etiology remains unknown. Factors such as family history and lifetime exposure to endogenous estrogens account for ~30% of breast cancer cases (2). Studies suggest that exogenous genotoxic carcinogens present in our diet and the environment may contribute to human breast cancer (3). It has been suggested that the highest cancer risks may result from ingestion of fried beef and fish products (4–6). 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), the most prevalent of the heterocyclic amines formed during the cooking process of various meats (4,7), is a mutagen in Salmonella typhimurium (8) and Chinese hamster ovarian cells (9,10). In vivo PhIP has been shown to induce both colon and mammary tumors in rodents (11,12). Although most studies have focused on the role of PhIP as a colon carcinogen, it also should be important to determine its potential involvement in human mammary tumor initiation.

Several recent studies suggest that normal human mammary epithelial (HME) cells may have the capacity to activate several carcinogens, one being PhIP, to species capable of binding DNA (13–15). In order for PhIP to bind to DNA, it must first be N-hydroxylated by cytochrome P450 1A2 (16,17). Furthermore, the N-hydroxylated metabolite is then converted to its ultimate carcinogen via phase II metabolizing enzymes, most prominently the acetyltansferases or sulfotransferases (15,18–26).

The finding that HME cells express only one of multiple isoforms of the sulfotransferase family, i.e. estrogen sulfotransferase (EST) (27), was of particular interest. This isoform has substrate specificities similar to that of the phenol form of sulfotransferase (P-PST) (28), previously shown to activate N-hydroxy PhIP (N-OH-PhIP) (21,25,26). The objective of this study was to determine the ability of EST to sulfate N-OH-PhIP and two other cooked food mutagens, N-hydroxy-2-amino-3-methylimidazo[4,5-f]quinoline (N-OH-IQ) and N-hydroxy-2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (N-OH-MeIQx), to species capable of binding to DNA. These studies were carried out both in vitro, using human recombinant EST, and in cultured HME cells.

[1H]N-OH-PhIP (101 mCi/mmol) (20), [1H]N-OH-IQ (99 mCi/mmol) and [1H]N-OH-MeIQx (130 mCi/mmol) were prepared as previously described (19,29). Ultrapure 3'-phosphoadenosine-5'-phosphosulfate (PAPS) was purchased from S.S. Singer (University of Dayton, Dayton, OH). [3H]PhIP (2.15 Ci/mmol) was obtained from Chemsys Science Laboratories (Lenexa, KS) through the National Cancer Institute Chemical Carcinogen Reference Standard Repository. All other chemicals were purchased from Sigma (St Louis, MO).

Recombinant human EST was isolated and purified from EST/pKK233-2 XL1-Blue cultures after induction with 1 mM isopropyl-β-D-thiogalactopyranoside for 5 h at 37°C, as previously described (28) with several modifications (30). The isolated enzyme preparation was free of other sulfotransferases but did contain bacterial proteins. Purification of EST to homogeneity was unsuccessful due to instability of the enzyme. The actual concentration of EST is therefore not known. It sulfated its natural substrate β-estradiol with a Ke value of 25 nM, which is similar to a previous report (28).

The EST-catalyzed sulfation of the N-hydroxylated amines was measured as the PAPS-dependent covalent binding to calf thymus DNA of the labile sulfenic acid ester formed, using radiolabeled substrates, as previously described for P-PST.
(26). Conditions for the reaction were optimized using 20 nM [3H]β-estradiol (28). Saturating concentrations of co-substrate PAPS were reached at 20 µM PAPS. Sulfation was linear with time up to 60 min and with enzyme up to 6 µL EST preparation.

The typical reaction mixture consisted of calf thymus DNA (2 mg/ml), recombinant human EST and 20 µM PAPS in a final incubation volume of 100 µl 33 mM Tris buffer (pH 7.5). The reactions were initiated with [3H]N-OH-PhIP, [3H]N-OH-IQ or [3H]N-OH-MeIQx (1, 10 and 100 µM) and were incubated at 37°C for 30 min under argon saturation. The radiolabeled drugs were added in dimethyl sulfoxide:ethanol (4:1). Control incubates did not contain the co-substrate PAPS. After incubation, the samples were extracted twice with n-butanol saturated with distilled water and once with phenol:chloroform:isoamyl alcohol (25:24:1, pH 8) (Ameresco, Solon, OH) with 1 g/l 8-hydroxyquinoline. DNA was then precipitated with 5 M sodium acetate and 100% ethanol. The DNA pellet was washed three times with ethanol, resuspended in Tris buffer and analyzed by liquid scintillation spectrometry. Washes were counted to ensure removal of non-specific binding. DNA recovery was determined by measuring the UV absorbance at 260 nm. On average, 90% of the DNA was recovered.

Primary HME cells, at passage 7, were obtained from Clonetics (San Diego, CA). These cells were derived from a 22-year-old healthy woman who had undergone reduction mammoplasty. Cell cultures were maintained as recommended by Clonetics. Serum-free mammary epithelial growth medium (HEPES-buffered balanced salt solution (HBSS) of the following composition: 10 mM glucose, 20 mM HEPES, 1.2 mM Na2HPO4, 1.2 mM MgSO4, 145 mM NaCl, 5 mM KCl, 2 mM CaCl2 and NaOH to a pH of 7.4. The cells were then incubated for 4 h at 37°C with 10 µM [3H]N-OH-PhIP in 5 ml HBSS in the presence or absence of 1 µM estrone. The incubation buffer was removed and cells were lifted by incubating them for 5 min at 37°C in 10 mM Tris buffer (pH 8) containing 1 mM EDTA and 0.14 M NaCl. Cells were collected by centrifugation and incubated overnight at 37°C in 10 mM Tris buffer (pH 8) containing 0.1 M EDTA, 100 µg/ml protease K and 0.5% SDS. The mixture was then extracted with 3 vols phenol:chloroform:isoamyl alcohol. DNA and RNA present in the aqueous phase were precipitated with 1 vol cold 100% ethanol and 5 M sodium acetate. The pellet was resuspended in 10 mM Tris buffer (pH 8) containing 0.1 M EDTA and 20 µg/ml pancreatic RNase and incubated for 1 h at 37°C. After extraction with 3 vols phenol:chloroform:isoamyl alcohol, DNA was again precipitated with 100% ethanol, collected by centrifugation and dissolved in water. DNA recovery was determined by UV absorbance at 260 nm. The amount of [3H]N-OH-PhIP bound to DNA was quantitated by liquid scintillation spectrometry.

Results are expressed as mean values ± SE. The statistical significance of differences was analyzed by Student’s t-test for unpaired data. Because of the non-normal distribution of the whole cell data, the Mann–Whitney test was used.

The EST-catalyzed binding of [3H]N-OH-PhIP to calf thymus DNA is shown in Figure 1. Figure 1A shows the binding of increasing concentrations of [3H]N-OH-PhIP to calf thymus DNA in incubates with 6 µL recombinant human EST. The open bars (Control) are in the absence of the co-substrate PAPS and the closed bars (+PAPS) in the presence of 20 µM PAPS. (B) Binding of 100 µM [3H]N-OH-PhIP to calf thymus DNA in incubates with increasing amounts of recombinant human EST. Each bar represents binding in the presence of 20 µM PAPS with the control binding, i.e. without PAPS, subtracted. Data are expressed as mean ± SE (n = 6). *Significantly higher than for the control (P < 0.001).

![Fig. 1](image-url)
than for the control (determined with 20 nM [3 H] by western blotting (27). The EST activity in HME cells, used intact cultured HME cells as the model system. EST is a potential oxidation products. It should be noted that the reactive species. It is of interest to notice that N-OH-MelQx and N-OH-IQ, mainly hepatocarcinogens (32,33), are bioactivated under more physiological conditions, we may better substrates for other bioactivating enzymes, such as the acetyltransferases (19,22). The high level of acetyltransferase activity in the liver may play a role in the tissue-specific carcinogenicity of IQ, the most potent rat and primate hepatocarcinogen described (22,33). Conversely, the higher affinity of N-OH-PhIP for EST expressed in normal breast cells may contribute to the tissue-specific carcinogenicity of PhIP in the mammary gland.

In order to assess the importance of EST-catalyzed N-OH-PhIP bioactivation under more physiological conditions, we used intact cultured HME cells as the model system. EST is the only sulfotransferase expressed in these cells, as determined by western blotting (27). The EST activity in HME cells, determined with 20 nM [3 H]-estradiol as substrate at 6 days after cell seeding, was 5.1 ± 1.0 pmol/mg cellular protein for a 3 h incubation, which is 2–3 times that reported previously at 1 day after cell seeding (27). To be able to use this model system in our studies, a specific EST inhibitor was obligatory. Estrone, by virtue of being another high affinity substrate for EST, provided this tool, producing virtually complete inhibition of β-estradiol sulfation by HME cells at a concentration as low as 1 μM. Using an established human red blood cell assay (34), estrone did not inhibit NAT-1, the other potential bioactivating enzyme (13), even at a concentration of 1000 μM.

To assess EST-dependent binding of [3 H]N-OH-PhIP to native DNA of the intact cell, HME cells were treated with 10 μM [3 H]N-OH-PhIP, the intermediate concentration in Figure 1A, for 4 h in the presence and absence of 1 μM estrone. Cellular DNA was then isolated and purified as described in Materials and methods and analyzed for incorpora-
tion of radiolabeled N-OH-PhIP. Figure 3A shows the EST-catalyzed binding of [3 H]N-OH-PhIP to native DNA of HME cells in eight individual experiments. In all experiments estrone reduced binding of N-OH-PhIP to DNA. Mean binding in the presence of the inhibitor was 55% of control (P < 0.01, n = 8). The variability between individual experiments appears to reflect a difference in EST expression. Thus, in experiments 5–8, performed in the same batch of cells at 6, 8, 9 and 10 days post-cell seeding, there was an EST-mediated binding of 11.8, 29.0, 58.8 and 160.0 pmol [3 H]N-OH-PhIP/mg DNA, respectively, i.e. markedly increased binding with increased maturity of the HME cells. A separate set of experiments examined EST expression in the HME cells at various times post-cell seeding. As shown in Figure 3B, there was an almost linear increase in EST activity over days 6–10, correlating well with the increased estrone-inhibitable DNA binding of [3 H]-estradiol as substrate.

Sadrieh et al. (15), using the [32P]-post-labeling technique, have indeed demonstrated with human mammary gland cytosol, although not with intact HME cells, that N-acetyltransferase 1 (NAT-1) O-acetylates N-OH-PhIP, increasing its reactivity towards DNA. It therefore appears feasible to postulate that both EST and NAT-1 present in normal human breast cells may further bioactivate N-OH-PhIP, promoting DNA adduct formation that, if unrepaired (13), may lead to tumor initiation.

It is not known if these cooked food heterocyclic amines are hydroxylated in the liver and then transported via the blood to their target sites for further enzymatic activation or if both oxidation and phase II esterification occur at the site of tumorigenicity. Whereas PhIP is the promutagen ingested with cooked foods (8), N-OH-PhIP is derived mainly from cytochrome P450 1A2 oxidation in the body. Although most of this oxidation probably takes place in the liver (16,19), it may also occur in breast cells. It has been suggested that DNA adducts are formed in HME cells when the cells are exposed to the promutagen PhIP (14). Although cytochrome P450 1A2 is not expressed in breast cells, a variety of other cytochrome P450 isoforms have recently been detected (35). In fact, a recent report shows that PhIP is a substrate for cytochrome P450 1B1, a newly described P450 present in normal human breast cells (36).

However, even if the generation of N-OH-PhIP from PhIP is not efficient in breast cells, N-OH-PhIP could be transported from the liver. Further bioactivation could then occur in the breast cells, as previously suggested (13). Although this might include O-acetylation (13,15), our present study suggests that this bioactivation also includes sulfation mediated by EST.

**Figure 1A, for 4 h in the presence and absence of 1 μM estrone. The EST activity in HME cells, determined with 20 nM [3 H]-estradiol as substrate at 6 days after cell seeding, was 5.1 ± 1.0 pmol/mg cellular protein for a 3 h incubation, which is 2–3 times that reported previously at 1 day after cell seeding (27). To be able to use this model system in our studies, a specific EST inhibitor was obligatory. Estrone, by virtue of being another high affinity substrate for EST, provided this tool, producing virtually complete inhibition of β-estradiol sulfation by HME cells at a concentration as low as 1 μM. Using an established human red blood cell assay (34), estrone did not inhibit NAT-1, the other potential bioactivating enzyme (13), even at a concentration of 1000 μM. To assess EST-dependent binding of [3 H]N-OH-PhIP to native DNA of the intact cell, HME cells were treated with 10 μM [3 H]N-OH-PhIP, the intermediate concentration in Figure 1A, for 4 h in the presence and absence of 1 μM estrone. Cellular DNA was then isolated and purified as described in Materials and methods and analyzed for incorporation of radiolabeled N-OH-PhIP. Figure 3A shows the EST-catalyzed binding of [3 H]N-OH-PhIP to native DNA of HME cells in eight individual experiments. In all experiments estrone reduced binding of N-OH-PhIP to DNA. Mean binding in the presence of the inhibitor was 55% of control (P < 0.01, n = 8). The variability between individual experiments appears to reflect a difference in EST expression. Thus, in experiments 5–8, performed in the same batch of cells at 6, 8, 9 and 10 days post-cell seeding, there was an EST-mediated binding of 11.8, 29.0, 58.8 and 160.0 pmol [3 H]N-OH-PhIP/mg DNA, respectively, i.e. markedly increased binding with increased maturity of the HME cells. A separate set of experiments examined EST expression in the HME cells at various times post-cell seeding. As shown in Figure 3B, there was an almost linear increase in EST activity over days 6–10, correlating well with the increased estrone-inhibitable DNA binding of [3 H]-estradiol as substrate.

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It is known that PhIP induces mutations in a tumor suppressor gene, APC, associated with colorectal cancer development (7,37). While mutations in various genes, including ras and p53, have been identified in PhIP-induced mammary tumors (38,39), the principal genes containing mutations are still an area of investigation.

EST is the only human sulfotransferase that has been shown to be hormonally regulated. Using an endometrial adenocarcinoma cell line, Falany and Falany found that progesterone increases EST expression as much as 7-fold (40). During the luteal phase of the menstrual cycle there is a surge in progesterone levels and one may speculate that during this time women may be more susceptible to carcinogen bioactivation through EST.

In conclusion, human EST has been shown to be capable of sulfocojugating N-OH-PhIP. This reaction was shown to increase the binding of N-OH-PhIP to calf thymus DNA by >3-fold. Extending these studies to the more physiological intact cultured HME cells clearly demonstrated EST-catalyzed binding of N-OH-PhIP to native cellular DNA. As EST is the only sulfotransferase isoform expressed in normal human breast cells, we hypothesize that this reaction significantly contributes to the initiation of breast cancer in humans.

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