ABSTRACT

Lactoperoxidase-catalyzed metabolism of N-hydroxy-N-2-fluorenylacetamide (N-OH-2-FAA) may be via one-electron oxidation to nitroso free radical which dismutates to equimolar N-acetoxy-N-2-fluorenylacetamide and 2-nitrosofluorene (2-NOF) and/or a Br⁻-dependent oxidative cleavage to 2-NOF. Hence, the 2-NOF:N-acetoxy-N-2-fluorenylacetamide ratios reflect the relative contributions of the two peroxidative pathways to the metabolism of N-OH-2-FAA. Peroxidative activities of rat uterus (UT) and mammary gland (MG) were extracted with a cationic detergent, cetlytrimethylammonium bromide (Cetab). MG extracts had 1 to 5% the specific activity of UT extracts when assayed with guaiacol as hydrogen donor. At 0.004% Cetab, which in the incubation media corresponds to the approximate physiological levels of 0.1 mM Br⁻, oxidation of N-OH-2-FAA by UT extracts yielded a product ratio indicative of both peroxidative pathways with Br⁻-dependent oxidation prevailing. At 0.4% Cetab, one-electron oxidation was negligible and Br⁻-dependent conversion of N-OH-2-FAA to 2-NOF was markedly enhanced. At both 0.004 and 0.04% Cetab, MG extracts yielded only 2-NOF, suggesting solely Br⁻-dependent oxidation. With equivalent guaiacol units of peroxidative activities, MG extracts produced much lower amounts of 2-NOF than did UT extracts. The low specific activities of MG extracts necessitated the use of larger amounts of protein, which might have interfered with peroxidative metabolism. At 0.004% Cetab, formation of 2-NOF by the Br⁻-dependent pathway was greater at pH 5.5 than at 7.4. At acid pH, small amounts of 2-nitrofluorene were also formed by UT and MG extracts and could be attributed to further oxidation of 2-NOF. Peroxidative activities of the UT and MG extracts may be of granular leukocyte origin and their potential role in carcinogen activation and tumorigenesis is discussed.

INTRODUCTION

Peroxidase-mediated oxidation of poly cyclic aromatic hydrocarbons (1, 2), aromatic amines (3–6), and estrogens (7–10) may be involved in their carcinogenicities, especially in extrahepatic tissues. Peroxidative oxidations of carcinogens have been described in a model system of HRP:H₂O₂ and also with a variety of mammalian peroxidases, including PES (reviewed in Refs. 1, 2, 11, and 12). They usually yield electrophilic reactants capable of binding covalently to nucleic acids, which is considered critical in initiation of carcinogenesis (13).

HRP and the mammalian peroxidase LPO and the hemoproteins cytochrome c and hematin, catalyzed 1e⁻ oxidation of carcinogenic N-arylhydroxamic acids (14–20). This oxidation for N-OH-2-FAA was via N-Ö-2-FAA which dismutated to equimolar N-AcO-2-FAA and 2-NOF (14, 18, 20). Both compounds were carcinogenic in the rat by application to the MG (21, 22) or s.c. tissue (23–25) but, probably due to their labilities, were less active than N-OH-2-FAA. Both compounds, especially 2-NOF, were potent direct mutagens (25–28). Covalently bound adducts of N-AcO-2-FAA with DNA were identified (29). Similar adducts of 2-NOF have not been identified, but interaction with DNA can be inferred from the results of the mutagenicity assays (27).

N-OH-2-FAA may also be oxidized largely to 2-NOF by LPO or extract of UT peroxidative activity in an H₂O₂ and Br⁻-dependent reaction (30). The extent of conversion of N-OH-2-FAA to 2-NOF depended on the concentration of Br⁻ and was enhanced by cationic detergents. A nearly complete conversion of N-OH-2-FAA to 2-NOF by LPO or UT extract occurred rapidly in buffers containing 4% Cetab or 4% Cetac plus 100 mM KBr. Evidence was presented that the oxidizing species includes OBr⁻ (or HOBr) generated enzymically from Br⁻ by peroxidase:H₂O₂ complex (Compound I) or chemically from Br₂:NaOH. It was suggested that this involves oxidative cleavage of N-OH-2-FAA to 2-NOF via an intermediary N-bromo-N-2-fluorenylhydroxylamine. The present investigation was undertaken to show whether oxidation of N-OH-2-FAA to 2-NOF by LPO and peroxidative activities extracted from rat UT and MG occurs at physiological levels of Br⁻ and therefore may occur in vivo. Since under these conditions LPO and UT extract mediated both 1e⁻ and Br⁻-dependent oxidations of N-OH-2-FAA, the effects of Cetab concentration and pH on the relative extents of these two types of oxidation by LPO and tissue peroxidative activities in vitro were determined.

MATERIALS AND METHODS

Fluorenyl Compounds. 2-FAA, 2-FA, and 2-NO₂F (Aldrich Chemical Co., Milwaukee, WI) were purified as described previously (20) and had m.p.s of 196–198°C, 127–129°C, and 159–160°C, respectively. N-OH-2-FAA, m.p. 150–151°C (31), 2-NOF, m.p. 79–81°C (32), N-AcO-2-FAA, m.p. 111–112°C (33) were prepared by the published procedures. The IR and UV spectra of the compounds matched those of the authentic samples. The compounds were found to be pure by HPLC.

Reagents, Columns, and Solvents. Bovine serum albumin, LPO (bovine milk), H₂O₂, guaiacol, Trisma hydrochloride, Cetab, and arachidonic acid were from Sigma Chemical Co., St. Louis, MO. TMBD was from Aldrich. All other reagents were ACS grade from Fisher Scientific Co., Pittsburgh, PA, or Mallinckrodt, Inc., Paris, KY. Perisorb RP-2, 30–40 μm (E. Merck) for guard columns was from Alttech Associates, Inc., Deerfield, IL. Zorbax C₈ columns were from DuPont Co., Wilmington, DE, and C₁₈ extraction columns (Baker-10 SPE) were from J. T. Baker Chemical Co., Phillipsburg, NJ. All solvents used for chromatography were glass-distilled-HPLC grade from Matheson Coleman & Bell, Norwood, OH.

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2 To whom requests for reprints should be addressed, at VA Medical Center (151B), Building 31, 54th St. and 48th Ave. South, Minneapolis, MN 55417.

3 The abbreviations used are: HRP, horseradish peroxidase; PES, prostaglandin endoperoxide synthase; 1e⁻, one electron; LPO, lactoperoxidase; N-OH-2-FAA, N-hydroxy-N-2-fluorenylacetamide; N-Ö-2-FAA, nitroso free radical of N-OH-2-FAA; N-AcO-2-FAA, N-acetoxy-N-2-fluorenylacetamide; 2-NOF, nitrosofluorene; MG, mammary gland; UT, uterus; Cetab, cetlytrimethylammonium bromide; 2-FAA, N-2-fluorenylacetic acid; 2-FA, N-2-fluorenylamine; 2-NO₂F, 2-nitrofluorene; HPLC, high-performance liquid chromatography; TMBD, 3,5,3',5'-tetramethylbenzidine; HB₀, hemoglobin; GU, guaiacol unit; ESR, electron spin resonance; RF, retention time.

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Laboratory for Cancer Research, Veterans Administration Medical Center, Minneapolis 55417 (D. M-G., R. W. D.) and Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis 55455 (D. M.-G., C. L. R., J. M. S.) Minneapolis.
Maintenance of Rats. Female Sprague-Dawley rats (specific pathogen free) from Harlan Sprague-Dawley, Inc., Indianapolis, IN, were maintained for 1 week on Purina laboratory Chow and water ad libitum. At 50 to 58 days of age and about 160 g, they were sacrificed by decapitation. Food was withheld for 15 h before sacrifice.

Homogenization of UT and MG and Extraction of Peroxidative Activities. All buffers were cooled in ice and all procedures were carried out at 4°C unless specified otherwise. UT and MG from 3 to 12 rats were excised and immersed in 50 mM Tris-HCl buffer, pH 7.4, with 154 mM KCl. After UTs were freed of fat and mesentery and MGs of muscle and lymph nodes, the tissues were rinsed and minced. The minces were then rinsed thoroughly over a 0.2-mm nylon mesh with the buffer used for homogenization, 5 mM sodium phosphate, pH 7.4, with 250 mM sucrose and 1 mM EDTA. The UT and MG minces were suspended in 4 and 1 parts (w/w), respectively, of the above buffer and homogenized with a Polytron homogenizer (type PT 10, Kinematica, GmbH, Lucerne, Switzerland) three times with 5-s bursts (45-s intervals between the bursts) at speed setting 6. The homogenates were centrifuged at 600 × g for 10 min. The pellets were resuspended in 0.5 volume of buffer, rehomogenized, and centrifuged as above. These pellets, rehomogenized in another 0.5 volume of buffer, were combined with the above to give total homogenate and were centrifuged at 105,000 × g for 1 h. Removal of HbO and solubilization of peroxidase were according to procedures used for rat UT (34). The final extracts in 80 mM sodium phosphate buffer, pH 6.3, containing 20% glycerol and 0.02% Cetab, were stored at −80°C.

Assays of Protein and Peroxidative Activities. Protein was determined in the above extracts by the modified Lowry method (35) using sodium dodecyl sulfate (1% in 0.1 N NaOH) to circumvent interference by Cetab. The concentration of H$_2$O$_2$ was determined spectrophotometrically using ε 43.6 m$^{-1}$ cm$^{-1}$ at 240 nm (36). Assays of peroxidative activities were in 100 mM sodium phosphate buffer, pH 7.4, at room temperature. The oxidation of guaiacol (13 HIM) was initiated by the addition of 0.33 or 0.16 mM H$_2$O$_2$ to UT or MG extracts, respectively, and the increase in absorbance at 470 nm was measured for 0.5 min. A GU is the amount of enzyme (extract) giving a change of 1 absorbance unit/s (34). Unless specified otherwise, references to peroxidative activity pertain to that determined with guaiacol.

Assays of Deacetylase Activity in UT and MG Extracts. Deacetylase activity with acetaldehyde or 2-FAA was measured as described (37), except that 100 mM sodium phosphate buffer, pH 7.0, with or without 4% Cetab was used. The limits of detection were 0.02 mm aniline and 0.01 mm 2-FAA in the incubation media. Deacetylase activity in Cetab extracts with N-OH-2-FAA was assayed by HPLC monitoring of 2-FAA in incubations lacking H$_2$O$_2$. The limit of detection was 75 nm 2-FAA in the incubation media.

Measurement of Heme Content and HbO. Heme content was calculated from the absorbance difference (λ$_{max}$ − λ$_{min}$) of the CN$^−$ difference spectra taken in 100 mM sodium phosphate buffer, pH 7.4, assuming an ε value similar to that determined for LPO (ε 114 m$^{-1}$ cm$^{-1}$) under identical conditions. HbO was measured as its CO complex at 420 nm based on ε 154 m$^{-1}$ cm$^{-1}$ (38).

All spectrophotometric determinations were carried out with a Hitachi 110 A spectrophotometer (Hitachi Instruments, Inc., Mountain View, CA) using the peak valley sensor program or enzyme mode.

Assay of PES Activity. Cyclooxygenase activity of PES in the UT and MG extracts was determined by measuring O$_2$ uptake into arachidonic acid to a cuvette containing 100 mM sodium phosphate, pH 7.0 or 7.4, either without or with 0.002 or 0.4% Cetab, extract (0.002–0.2 GU, 0.2–0.8 mg protein) and 83 nmol N-OH-2-FAA in 1.0 ml total volume. TMBD or N-OH-2-FAA was omitted from the reference cuvette. Oxidation of TMBD or N-OH-2-FAA was measured by monitoring spectral changes from 700 to 260 nm (39) or 420 to 240 nm, respectively.

Determination of N-Ö-2-FAA by ESR Spectroscopy. Formation of N-Ö-2-FAA in incubations containing at least 0.024 GU LPO or UT extract, 0.025 to 0.08 mm N-OH-2-FAA (in 0.01 ml methanol), and 0.015 to 0.09 mm H$_2$O$_2$ in a total volume of 3.0 ml of 100 mM sodium phosphate buffer, pH 7.4, was monitored as described previously (20).

Determination of Metabolism of N-OH-2-FAA by LPO or Extracts of UT or MG Peroxidative Activities. The incubation mixtures usually consisted of 0.008 GU of peroxidative activity of LPO or tissue extract and 80 nmol N-OH-2-FAA in 0.01 ml methanol in a total volume of 1.0 ml of 100 mM sodium phosphate buffer, pH 7.4 or 5.5, containing Cetab at a given concentration. The reactions were started by additions of H$_2$O$_2$. Formation of 2-NOF at room temperature was monitored spectrophotometrically at 370 nm and quantitated using ε 18.4 m$^{-1}$ cm$^{-1}$ determined by adding 2-NOF to buffers. Control incubations contained no H$_2$O$_2$. At times indicated, the mixtures were transferred onto Baker C$_8$ extraction columns (1.0-ml capacity) which had been activated with 0.5 ml methanol followed by 0.5 ml 100 mM sodium phosphate buffer, pH 7.4. The columns were then washed with 0.5 ml of the above buffer. The reaction products were eluted with 0.5 ml of methanol:iso-propanol (1:1). The eluates were dried over Na$_2$SO$_4$ and evaporated under argon passed through Oxiclear (Pierce Chemical Co., Rockford, IL) at 30°C for 20 min to avoid vaporization of the compounds. The residues were dissolved in 0.05 to 0.1 ml methanol and 0.01-ml aliquots were analyzed by HPLC.

HPLC Analyses. All chromatographic analyses were carried out with a liquid chromatograph (Model 5060; Varian Instruments, Sunnyvale, CA) equipped with a pneumatically activated injector and a 0.01-ml loop (Valco Instruments, Houston, TX) and a Hewlett-Packard 1040A high speed spectrophotometric detector, a DPU multichannel integrator (79882A), a computer (855B), Thinkjet printer (2225A), and plotter (7470A) (Hewlett-Packard, Palo Alto, CA).

Two HPLC systems, A and B, were used. Guard columns preceded the analytical columns. System A consisted of a Zorbax C$_8$ column (150 × 4.6 mm inside diameter) operated at 30°C and a flow rate of 0.8 ml/min with an operating pressure of 1200 psi and a mobile phase of methanol:0.2 M acetic acid, pH 3.8 (2:1). The absorbance of the compounds was monitored at 230, 280, 330, and 360 nm (Fig. 1). For identification of the compounds their UV spectra were memorized from the analytical columns. System B consisted of a Golden Series Zorbax C$_8$ column (80 × 6.2 mm inside diameter) operated at 40°C and a flow rate of 3 ml/min, with an operating
pressure of 3200 psi and the mobile phase described previously (40). The absorbance of the eluate was monitored at 260, 280, and 300 nm. The UV spectra and the peak area of N-OH-2-FAA (Rf 9.42 min) were memorized and integrated at 280 nm. UV spectra of N-OH-2-FAA and its metabolites were superimposable with those of standard compounds chromatographed in System A or B. The compounds were quantified from peak areas relative to standard curves. The amounts were corrected for losses during extraction, transfer, and evaporation procedures, and for dilution of the extract. The losses were determined with mixtures of standard compounds incubated in matching reaction media without active extract or H2O2. The recoveries of the standard compounds were similar in both cases, but varied from 43 to 85%, depending on the compound itself and composition of the media. The low peroxidative activities of MG extracts necessitated using larger volumes. In these cases the recovery of N-AcO-2-FAA was about 20%, while that of 2-NOF remained at about 40%.

RESULTS

Characterization of Extracts of Peroxidative Activities from UT and MG. Since peroxidative activity was detected in nuclear, mitochondrial, and microsomal fractions, the entire particulate fraction of the UT or MG homogenate was sedimented and extracted. Washing with 250 mM NaCl removed HBO but decreased peroxidative activities of the extracts from 4 to 25%. Similar amounts of protein (mg/rat) were extracted from UT and MG, although the wet weight of MG (g/rat) was more than 10 times that of UT (Table 1). Specific activities of UT extracts were consistent among several tissue pools examined. The specific activities of the MG extracts fell into two ranges with 1 to 5% the specific activity of the UT extracts. Optimal pHs for the oxidation of guaiacol by UT and MG extracts were found to be 7.0 to 7.4. The cyclooxygenase activity of PES was undetectable in the UT and MG extracts by incorporation of molecular oxygen into arachidonic acid. However, trace TMBD (<1%) was oxidized in sodium acetate buffer, pH 5.0, by UT, but not MG, extract preincubated with arachidonic acid for 10 min at 37°C, suggesting marginal level of PES activity in the UT extract. Peroxidative activities of both UT and MG extracts were stable with storage at −80°C for up to 6 months. The ratios of GU to nmol of heme provided an estimate of the purity of the extracts. As determined from CN−-difference spectra, the ratios were 0.88 and 0.07 or 0.01 for UT and MG extracts, respectively (Table 1). A ratio of 4.5 has been reported for UT extract purified 50- to 250-fold (34).

Peroxidative Metabolism of N-OH-2-FAA by LPO. Depending on the composition of the reaction media, LPO may catalyze either 1e oxidation to N-O-2-FAA which then dismutates to equimolar 2-NOF and N-AcO-2-FAA (15, 41) or Br−-dependent oxidative cleavage to 2-NOF (30). Hence, the 2-NOF:N-AcO-2-FAA ratio varied with the peroxidase system used (16, 20). In 0.004% Cetab (Table 2) showed a 2-fold increase in the formation of 2-NOF at pH 5.5, but its overall capacity for peroxidative metabolism of N-OH-2-FAA was only about 20% of UT extracts from Experiment 1. This variability among extracts may result from different reactivity of the extracted peroxidase(s) with N-OH-2-FAA compared to guaiacol. At pH 5.5 and 0.004% Cetab, 2-NO2F was also formed, most likely from further oxidation of 2-NOF, which has been found to be 7.0 to 7.4. The cyclooxygenase activity of PES was undetectable in the UT and MG extracts by incorporation of molecular oxygen into arachidonic acid. However, trace TMBD (<1%) was oxidized in sodium acetate buffer, pH 5.0, by UT, but not MG, extract preincubated with arachidonic acid for 10 min at 37°C, suggesting marginal level of PES activity in the UT extract. Peroxidative activities of both UT and MG extracts were stable with storage at −80°C for up to 6 months. The ratios of GU to nmol of heme provided an estimate of the purity of the extracts. As determined from CN−-difference spectra, the ratios were 0.88 and 0.07 or 0.01 for UT and MG extracts, respectively (Table 1). A ratio of 4.5 has been reported for UT extract purified 50- to 250-fold (34).

Peroxidative Metabolism of N-OH-2-FAA by UT Extracts. The extent of conversion of N-OH-2-FAA to 2-NOF by UT extracts (measured spectrophotometrically) was shown to depend on Cetab and H2O2 concentrations in the incubation media (Fig. 2). As with LPO, at 0.1 mM H2O2 and 0.4% Cetab, the conversion to 2-NOF was nearly complete in 14 min at room temperature. Arachidonic acid did not substitute for the H2O2 in this conversion. However, 2-NO2F could contribute to this absorbance. To examine this, extracts of the incubation mixtures in 0.4% Cetab were analyzed by HPLC and compared to those in 0.004% Cetab, corresponding to physiological levels of 0.1 mM Br− (42) and possible low pH (43, 44) (Table 3). In all experiments the formation of 2-NOF increased with time and with increasing Cetab concentration. The amounts of 2-NOF quantified by the HPLC method corresponded to the amounts determined spectrophotometrically. At 0.004% Cetab, UT extracts in Experiment 1 formed substantial amounts of N-AcO-2-FAA with 2-NOF:N-AcO-2-FAA ratios of 3 to 7 at pH 7.4. This suggested contribution of 1e oxidation to overall peroxidative metabolism of N-OH-2-FAA. Its contribution was less with the UT extracts in Experiment 2 as shown by ratios of 30, and was negligible at 0.4% Cetab as indicated by ratios >100 in Experiment 3; 1e oxidation was also decreased at pH 5.5 as indicated by the ratios of 9 to 14 in Experiment 1 and by the almost 100% conversion to 2-NOF in Experiment 3. Another UT extract in 0.004% Cetab (not included in Table 3) showed a 2-fold increase in the formation of 2-NOF at pH 5.5, but its overall capacity for peroxidative metabolism of N-OH-2-FAA was only about 20% of UT extracts from Experiment 1. This variability among extracts may result from different reactivity of the extracted peroxidase(s) with N-OH-2-FAA compared to guaiacol. At pH 5.5 and 0.004% Cetab, 2-NO2F was also formed, most likely from further oxidation of 2-NOF, since a 5% conversion of 2-NOF to 2-NO2F by the UT extracts under these conditions was determined (data not shown). In all experiments small amounts of 2-FAA were determined among the metabolites of N-OH-2-FAA formed by UT extracts (Table 3). The amounts of amide by-products have been reported to vary with the peroxidase system used (16, 20). In 0.4% Cetab media, in the absence of enzyme, <0.15% N-AcO-2-FAA was converted to 2-FAA (data not shown), suggesting that only a small fraction of 2-FAA could have come from Br−-dependent reduction of N-AcO-2-FAA (45).

Peroxidative Metabolism of N-OH-2-FAA by MG Extracts. Preliminary results with MG extracts (0.004 GU) and 0.01%
Peroxidative metabolism of N-OH-2-FAA

Table 2. Metabolism of N-OH-2-FAA by LPO

Incubation mixtures contained 0.008 GU of LPO and 77 to 83 mmol N-OH-2-FAA in 0.01 ml methanol in a total volume of 1.0 ml of 100 mm sodium phosphate buffer, pH 7.4, containing Cetab with or without KBr and H₂O₂ at the concentrations indicated below. Control incubations contained no H₂O₂. After incubations for 8 min at room temperature, N-OH-2-FAA and its metabolites were extracted and analyzed by HPLC as described in "Materials and Methods."

<table>
<thead>
<tr>
<th>Incubation</th>
<th>Metabolites (nmol)</th>
<th>Recovered N-OH-2-FAA (nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cetab (%)</td>
<td>KBr (mm)</td>
<td>H₂O₂ (mm)</td>
</tr>
<tr>
<td>0.004</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.004</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.004</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
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<td>1</td>
<td>-</td>
</tr>
<tr>
<td>0.004</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>0.004</td>
<td>100</td>
<td>0.1</td>
</tr>
<tr>
<td>0.4</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* No metabolites were detected in the control incubations.
+ —, no Cetab; in this experiment 0.024 GU of LPO was used.
—, no KBr.
Mean ± SD from 2 experiments, each carried out in duplicate and corrected for recoveries determined with the standard compounds.

This experiment at pH 5.5.

Fig. 2. Formation of 2-NOF from N-OH-2-FAA by extracts of UT peroxidative activity in buffers containing Cetab and in the presence of H₂O₂. UT extracts were prepared as described in "Materials and Methods." Incubation mixtures consisted of 0.008 GU (0.05 to 0.09 mg protein) of the UT extract (0.015 ml in 0.02% Cetab), 63 nmol of N-OH-2-FAA in 0.008 ml methanol in a total volume of 1.0 ml of 100 mm sodium phosphate buffer, pH 7.4 (○) or the buffer containing 0.02% Cetab (■) or 0.4% Cetab (□). The reaction was started by addition of H₂O₂ at concentrations indicated. The formation of 2-NOF was measured spectrophotometrically as described in "Materials and Methods." The spectra between 400 and 330 nm were recorded at 2-min intervals and 2-NOF was calculated when absorbance at 370 nm was maximal.

Cetab in the incubation mixture showed a 5% conversion of N-OH-2-FAA to 2-NOF with no formation of N-AcO-2-FAA (41). Using 0.008 GU of MG extract, the peroxidative metabolism of N-OH-2-FAA was now examined in 0.004 and 0.4% Cetab and compared to that by UT extracts. In contrast to UT extracts (Table 3), MG extracts did not form detectable N-AcO-2-FAA and 2-NOF (14) and/or oxidative cleavage to 2-FAA (Table 4). Since its formation depended on H₂O₂, it was suspected to arise from the metabolite 2-NOF. Indeed, 1 to 5% 2-FA was formed from 2-NOF incubated with MG extracts in the presence or absence of H₂O₂ (data not shown). This suggested that 2-NOF-reducing activity is inherent to the MG extract and is independent of the peroxidative pathway. Deacetylation of N-OH-2-FAA and/or 2-FAA by MG extracts was ruled out as a potential source of 2-FA since no amine was formed from N-OH-2-FAA in the absence of H₂O₂ and no deacetylase activity was measured by a classical procedure (37).

DISCUSSION

Peroxidative metabolism of N-OH-2-FAA may be via 1e⁻ oxidation to N-O₂-2-FAA which dismutates to equimolar N-AcO-2-FAA and 2-NOF (14) and/or oxidative cleavage to 2-NOF (30). Whereas in 1e⁻ oxidation, N-OH-2-FAA is oxidized by the peroxidase:H₂O₂ complex (Compound 1), in the other peroxidative pathway, Compound 1 oxidizes Br⁻ to OBr⁻ which in turn oxidizes N-OH-2-FAA (30) (Fig. 3). LPO is a mammalian peroxidase capable of catalyzing both peroxidative pathways with N-OH-2-FAA, depending on the composition of the reaction medium (15, 30, 41). Probably because N-OH-2-FAA is oxidized more slowly than Br⁻ by Compound 1, demonstration of 1e⁻ oxidation requires much lower concentrations of H₂O₂ than Br⁻-dependent oxidation of N-OH-2-FAA (30, 41) (Table 2). The latter oxidation was enhanced by cationic detergents, but the mechanism of this enhancement has not been determined.

The low concentration of Cetab (0.004%) used in this study was chosen to mimic the estimated average physiological concentration of Br⁻ (~0.1 mm) in body fluids (42). Cetab was also essential for maintaining solubility of the peroxidative activities of UT and MG. Use of 0.5 M CaCl₂ for this purpose decreased the reaction rate by 2- to 3-fold at pH 7.4, but not any further at pH 5.5. At both pHs, only trace amounts of 2-NO₂F were detected. Thus, factors other than Cetab concentration limit the oxidation rate at the lower pH. In general, MG extracts had about 10% the capacity of UT extracts to metabolize N-OH-2-FAA to 2-NOF. Since the specific activities of peroxidative enzyme(s) were 20 to 85 times lower in the MG than in the UT extracts, the associated protein(s) might have interfered with peroxidative metabolism.

As with UT extracts, 2-FA was a by-product of peroxidative metabolism of N-OH-2-FAA by MG extracts. Unlike the UT extracts, MG extracts formed small amounts of 2-FA (Table 4). Since its formation depended on H₂O₂, it was suspected to arise from the metabolite 2-NOF. Indeed, 1 to 5% 2-FA was formed from 2-NOF incubated with MG extracts in the presence or absence of H₂O₂ (data not shown). This suggested that 2-NOF-reducing activity is inherent to the MG extract and is independent of the peroxidative pathway. Deacetylation of N-OH-2-FAA and/or 2-FAA by MG extracts was ruled out as a potential source of 2-FA since no amine was formed from N-OH-2-FAA in the absence of H₂O₂ and no deacetylase activity was measured by a classical procedure (37).
PEROXIDATIVE METABOLISM OF N-OH-2-FAA

Table 3: Metabolism of N-OH-2-FAA by extracts of UT peroxidative activity

<table>
<thead>
<tr>
<th>Incubation</th>
<th>Metabolites (nmol)*</th>
<th>Recovered N-OH-2-FAA (nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment</td>
<td>2-FAA</td>
<td>N-AcO-2-FAA</td>
</tr>
<tr>
<td>1</td>
<td>0.004</td>
<td>7.4</td>
</tr>
<tr>
<td>2</td>
<td>0.004</td>
<td>7.4</td>
</tr>
<tr>
<td>3</td>
<td>0.4</td>
<td>7.4</td>
</tr>
<tr>
<td>5.5</td>
<td>8</td>
<td>0.25 ± 0.06</td>
</tr>
</tbody>
</table>

* No metabolites were detected in the control incubations from which 82.8 ± 8.5 nmol of N-OH-2-FAA were recovered.

Table 4: Metabolism of N-OH-2-FAA by extracts of MG peroxidative activity

<table>
<thead>
<tr>
<th>Incubation</th>
<th>Metabolites (nmol)*</th>
<th>Recovered N-OH-2-FAA (nmol)</th>
</tr>
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<tbody>
<tr>
<td>Experiment</td>
<td>2-FAA</td>
<td>2-FAA</td>
</tr>
<tr>
<td>1</td>
<td>0.004</td>
<td>7.4</td>
</tr>
<tr>
<td>2</td>
<td>0.4</td>
<td>7.4</td>
</tr>
<tr>
<td>5.5</td>
<td>8</td>
<td>&lt;0.15</td>
</tr>
</tbody>
</table>

* No metabolites were detected in the control incubations from which 80.0 ± 6.5 nmol of N-OH-2-FAA were recovered.

Fig. 3: Suggested mechanism of oxidation of N-OH-2-FAA by Br⁻-oxidizing peroxidase. Evidence for oxidative cleavage was provided with the use of \(^{14}C\)-labeled N-OH-2-FAA (30). * C denotes \(^{14}C\).

by Ca²⁺ (41). At 0.004% Cetab, oxidation of N-OH-2-FAA by UT extracts yielded N-AcO-2-FAA and 2-NOF in ratios indicative of both peroxidative pathways with the Br⁻-dependent oxidation prevailing (Table 3), whereas oxidation by MG extracts yielded only 2-NOF suggesting solely the Br⁻-dependent oxidation (Table 4). MG extracts produced much lower amounts of 2-NOF than did UT extracts, even though equivalent GUs of peroxidative activities were used. The low specific activities of MG extracts necessitated using greater amounts of protein in the incubations, which may have interfered with peroxidative metabolism of N-OH-2-FAA. At higher concentrations of Cetab (0.4%), 1e⁻ oxidation of N-OH-2-FAA by UT extracts was negligible. The trace to small amounts of 2-NO₂F formed by both UT and MG extracts at pH 5.5 and 0.004% Cetab are attributed to further oxidation of 2-NOF since they were produced with 2-NOF as the substrate. In contrast to UT, MG extracts also reduced 2-NOF as shown by formation of 2-FA which was independent of peroxidative activity. A relatively strong reducing activity of MG microsomal and mitochondrial fractions was reported previously (46, 47).

Generation of N-Ö-2-FAA as an obligatory intermediate in 1e⁻ oxidation of N-OH-2-FAA has been shown by ESR spectroscopy with various hemoproteins (14–20). However, the only evidence of ESR signals generated from N-OH-2-FAA by tissue or cell preparations containing peroxidative activity was from MG cells incubated with peroxides (48) or with arachidonic acid (49). In the present study, an ESR signal of N-Ö-2-FAA was obtained with LPO:H₂O₂, but only in the phosphate buffer without Cetab. Addition of as little as 0.001% Cetab to LPO prior to the incubation with H₂O₂ prevented the detection of N-Ö-2-FAA. On the other hand, addition of Cetab to the solution containing N-Ö-2-FAA did not quench the radical. This suggests that interaction of the protein and Cetab prevents the radical detection. For the same reason an ESR signal of N-Ö-2-FAA was undetectable with UT extracts in 0.004% Cetab, even though the extent of le⁻ oxidation appeared sufficient for the radical detection.
On the other hand, the very low PES activity detected in the UT extracts with TMBD suggests that a small fraction of peroxidative metabolism of N-OH-2-FAA by these extracts could be due to peroxidase activity of PES. Whether PES can oxidize N-OH-2-FAA has not been substantiated. The only report suggesting this showed partial inhibition by indomethacin or aspirin of the 2-NOF formation from N-OH-2-FAA by rat seminal vesicle microsomes (49). How the relatively large amounts of 2-NOF, presumably generated by PES, could be detected is unclear in view of the evidence presented by other investigators that PES rapidly oxidized 2-NOF to 2-NOF (6, 50). Like PES (3–6, 50, 51), peroxidative activities of rat UT and MG extracts in the presence of H2O2 catalyzed 1e- oxidation of aromatic amines (41). This suggests that the oxidation of carcinogenic N-arylamines could occur at these sites in vivo and lead to DNA binding and initiation of carcinogenesis.

Of several organs examined in normal rats, the UT had the greatest peroxidative activity (52), which appears largely of eosinophil origin (53–55). The presence of intracellular peroxidase, presumably LPO, has been shown in the rat MG only during late pregnancy and lactation (56, 57). Peroxidative activity of the MG from virgin rats has been reported (58–60) but its origin is unclear. It is possible that this activity, as that in UT, is of eosinophil origin. Eosinophil peroxidase catalyzed the Br- but not Cl- dependent decarboxylation of amino acids (61), and likewise the UT extracts utilized only Br- for the conversion of N-OH-2-FAA to 2-NOF (30). This suggests eosinophil origin of the peroxidative activity of the UT extracts.

It is thus possible that N-OH-2-FAA and related hydroxy acids are activated by peroxidases of granular leukocytes (eosinophils and/or neutrophils) which penetrate the tissues. Activation of N-arylamines by myeloperoxidase or by neutrophils treated with a phorbol ester to compounds binding to cell thymus or leukocyte DNA, respectively, has recently been reported (62). The peroxidases and H2O2 may also be released from leukocytes extracellularly, where in the presence of a halide they generate hypohalous acid (43, 63–65). The latter may oxidize N-arylnitrosamines by a mechanism exemplified with N-OH-2-FAA (Fig. 3). Consequent, large amounts of the nitroso compounds would be released in intercellular spaces wherefrom they may penetrate cell membranes. This would be facilitated by the relatively lipophilic nature of the nitroso aromatics (66). Subsequent reduction of the nitroso function would yield N-arylnitrosamines which bind to DNA per se or more preferably via N-acetoxyamines following O-acetylation by an acetyl CoA-dependent cytosolic enzyme (67). Although the nitroso compounds are highly reactive intermediates (32, 68–70), their status as potential ultimate carcinogens is as yet unclear. This requires further investigation particularly that 2-NOF was the most potent direct mutagen of all N-OH-2-FAA derivatives tested (27, 28, 71), and yet, as such, is unreactive with DNA (72). The DNA adducts isolated from UT and/or Br- dependent peroxidative conversion of N-OH-2-FAA to 2-NOF as reported herein. The DNA adducts from rat UT have not been characterized. The finding of this study that UT extract generates both N-AcO-2-FAA and 2-NOF argues in favor of in vivo formation of both types of adducts with UT DNA, N-acetylated and N-deacetylated. N-OH-2-FAA is a potent carcinogen after direct application to the MG (22, 76), but has not been tested in UT by the topical route. However, 2-FAA administered in diet produced endometrial carcinomas in the rabbit (77), the incidence of which was increased by treatment with estrogen. Dependency of UT peroxidative activity on estrogen has been shown (52–54).

REFERENCES


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PEROXIDATIVE METABOLISM OF N-OH-2-FAA


Peroxidative Metabolism of a Carcinogen, \(N\)-Hydroxy-\(N\)-2-fluorenylacetamide, by Rat Uterus and Mammary Gland \textit{in Vitro}
