Peroxynitrite Irreversibly Inactivates the Human Xenobiotic-metabolizing Enzyme Arylamine N-Acetyltransferase 1 (NAT1) in Human Breast Cancer Cells

A CELLULAR AND MECHANISTIC STUDY*  

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Arylamine N-acetyltransferases (NATs) play an important role in the detoxification and metabolic activation of a variety of aromatic xenobiotics, including numerous carcinogenic. Both of the human isoforms, NAT1 and NAT2, display interindividual variations, and associations between NAT genotypes and cancer risk have been established. Contrary to NAT2, NAT1 has a ubiquitous tissue distribution and has been shown to be expressed in cancer cells. Given that the activity of NAT1 depends on a reactive cysteine that can be a target for oxidants, we studied whether peroxynitrite, a highly reactive nitrogen species involved in human carcinogenesis, could inhibit the activity of endogenous NAT1 in MCF7 breast cancer cells. We show here that exposure of MCF7 cells to physiological concentrations of peroxynitrite and to a peroxynitrite generator (3-morpholinosydnonimine N-ethylcarbamate, or SIN1) leads to the irreversible inactivation of NAT1 in cells. Further kinetic and mechanistic analyses using recombinant NAT1 showed that the enzyme is rapidly \( k_{\text{inact}} = 5 \times 10^4 \text{ m}^{-1} \text{s}^{-1} \) and irreversibly inactivated by peroxynitrite. This inactivation is due to oxidative modification of the catalytic cysteine. We conclude that the reducing cellular environment of MCF7 cells does not sufficiently protect NAT1 from peroxynitrite-dependent inactivation and that only high concentrations of reduced glutathione could significantly protect NAT1. Thus, cellular generation of peroxynitrite may contribute to carcinogenesis and tumor progression by weakening key cellular defense enzymes such as NAT1.

Acetylation is a major biotransformation pathway for various aromatic amines, including drugs and carcinogens. Many arylamines, such as 4-aminobiphenyl (a tobacco-associated compound) and benzidine (occupational exposure) are important environmental carcinogens (1–3), and their biotransformation through \( N- \) and/or \( O- \) acetylation has been linked to carcinogenesis (2–5). In humans, these reactions are catalyzed by two xenobiotic-metabolizing enzymes (XME),\(^1\) arylamine \( N \)-acetyltransferase 1 and arylamine \( N \)-acetyltransferase 2 (NAT1 and NAT2; EC 2.3.1.5). Both of the human isoforms, NAT1 and NAT2, display interindividual variations and associations between NAT genotypes, and cancer risk has been established (1, 3, 5). In addition, these cytosolic enzymes are encoded by two separate genes located on 8p22, a chromosomal region commonly deleted in certain human cancers (5–7). This raised the possibility that the absence of NATs or their inactivation may contribute to carcinogenesis and/or tumor progression (6, 8, 9). Despite their high degrees of sequence identity, NAT1 and NAT2 differ markedly in terms of amine-containing acceptor substrates (10, 11) and tissue distribution (2, 12). Indeed, NAT2 is primarily located in the liver and colon epithelium (13, 14), whereas NAT1 seems ubiquitous (15, 16). In addition, NAT1 expression has been demonstrated in different cancers and especially in human breast cancer, where it may play a role in cancer progression (17).

NAT isoforms have been detected in a number of species, from bacteria to mammals (11, 18). Crystallographic determination of the structure of the NATs from \( \text{Salmonella typhi} \) murium and \( \text{Mycobacterium smegmatis} \), and the subsequent construction of theoretical models of human NAT1 and NAT2 revealed structural similarities to cysteine proteases (19–22). These data demonstrated the existence of a conserved cysteine protease-like catalytic triad (Cys, His, and Asp) in NATs (19–22), confirming the fundamental role in catalysis of a conserved active site cysteine residue (11, 23, 24). Thus, NATs may be prone to inactivation by oxidative reactions as shown recently for NAT1 (25) and for other reactive cysteine-containing enzymes (26–29).

Nitric oxide and reactive nitrogen species (RNS) are major biological oxidants. They have been implicated in major physiological and pathophysiological processes such as vasorelaxation, apoptosis, inflammation, and cancer through the oxidative modification of DNA, proteins, or lipids (30–33). Peroxynitrite \( \text{(ONOO}^{-}) \) is one of the most reactive and, therefore, most deleterious nitric oxide derivatives involved in the oxidative modification of biological molecules (32, 33). Peroxynitrite affects protein functions by modifying essential reactive thiols or tyrosine residues (32). For instance, it has been shown to irreversibly inactivate fundamental enzymes

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\( ^1 \) The abbreviations used are: XME, xenobiotic-metabolizing enzymes; NAT, arylamine \( N \)-acetyltransferase; SIN1, 3-morpholinosydnonimine \( N \)-ethylcarbamide; PAS, p-aminosalicylic acid; PNPA, p-nitrophenylacetate; AcCoA, acetyl-coenzyme A; DTT, 1,4-dithiothreitol; PBS, phosphate-buffered saline.
such as creatine kinase (34), tryptophan hydroxylase (35), caspases (36), and phosphatases (37). XMEs, such as cytochromes P450 (38, 39) and glutathione S-transferase (40), have also been reported to be irreversibly inactivated by peroxynitrite.

Peroxynitrite generation has been demonstrated during sepsis as well as in autoimmune and inflammatory conditions (32, 41). In addition, evidence is accumulating from studies in vitro and in vivo that peroxynitrite is generated during carcinogenesis and promotes this process, in particular for colon and breast cancer (30, 42–44). As stated above, NAT1 has been shown to be highly expressed in normal and cancerous human breast tissue (17). Therefore, we decided to investigate whether peroxynitrite could inhibit endogenous NAT1 in MCF7 breast cancer cells.

Using peroxynitrite and the peroxynitrite generator 3-morpholinosydnonimine N-ethylcarbamide (SIN1), we investigated the effect of peroxynitrite on the activity of NAT1 in human breast cancer cells. We found that exposure of cultured MCF7 cells to physiological concentrations of peroxynitrite or SIN1 for a short period of time (10–30 min) led to the irreversible inactivation of the endogenous NAT1 enzyme. Further mechanistic analyses of the reaction of peroxynitrite with purified recombinant NAT1 showed that this XME is very rapidly (k_{inact} \approx 5 \times 10^{10} \text{ M}^{-1} \text{s}^{-1}) and irreversibly inactivated by physiological concentrations of peroxynitrite. In addition, we showed that the peroxynitrite-dependent inactivation of NAT1 was due to irreversible oxidative modification of the catalytic cysteine residue of the enzyme. Thus, our results suggest that NAT1 activity may be regulated in vivo by nitrosative stress, with potentially important implications in carcinogenesis and tumor progression.

MATERIALS AND METHODS

Materials—Peroxynitrite and SIN1 hydrochloride were obtained from Calbiochem-Novabiochem, and their chemical structures are shown in Fig. 1. p-Aminosalicylic acid (PAS), p-nitrophenylacetate (PNPA), acetyl-coenzyme A (AcCoA), coenzyme A (CoA), 1,4-dithiothreitol (DTT), and reduced glutathione (GSH) were obtained from Sigma. The vector, pET28, was purchased from Novagen. Nickel-nitritotriacetic acid Superflow resin was obtained from Qiagen. Anti-fluorescein Fab fragments conjugated to peroxidase, fluorescein-conjugated iodoacetamide, and Complete protease inhibitor tablets were obtained from Roche Applied Science. The Bradford protein assay kit was supplied by Bio-Rad. All other reagents were purchased from Sigma or Eurobio. Polyclonal antibody against human NAT1 was a kind gift of Prof. Edith Sim (University of Oxford, Oxford, UK).

Cell Culture, Peroxynitrite/SIN1 Treatment, and Total Cell Extracts—MCF7 cells (human breast carcinoma) were cultured as monolayers in 100-mm Petri dishes at 37 °C in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 20% (v/v) fetal bovine serum and penicillin/streptomycin. At ~90% confluence, cell monolayers were washed with PBS (Ca^{2+}-Mg^{2+}). Cell monolayers were exposed to different concentrations of peroxynitrite or SIN1 in 10 ml of PBS and kept for 10 min (peroxynitrite) or 30 min (SIN1) at 37 °C. Control treatments were performed with decomposed SIN1 (obtained by allowing decomposition at room temperature in the dark for 48 h) or with PBS only. After treatment, monolayers were washed with PBS, scraped in 1 ml of lysis buffer (20 mM Tris-HCl, 1 mM DTT, pH 7.5, 0.2% Triton X-100, and protease inhibitors) and centrifuged for 1 h at 100,000 × g. Supernatants (total cell extracts) were taken, and protein concentrations were determined using the Bradford method. All cell extracts were adjusted to the same protein concentration by adding 20 mM Tris-HCl and 1 mM DTT, pH 7.5, and then used in the experiments described below.

Detection of Endogenous NAT1 Protein in MCF7 Cell Extracts—To assess the amount of NAT1 in MCF7 cell extracts, all NAT1 protein present in these extracts was immuno precipitated for 2 h at 4 °C using a saturating amount of purified anti-NAT1 antibody (1 μg of IgG fraction) in a total volume of 300 μl. Equal amounts of protein A-agarose were then added, and the mixture was rocked for 2 h at 4 °C. Beads were then washed twice with PBS. Bound NAT1 was eluted by boiling in non-reducing SDS sample buffer and subjected to SDS-PAGE and Western blotting using the anti-NAT1 antibody.

Production and Purification of Recombinant Human NAT1—The human NAT1 cDNA was subcloned into pET28. This construct was used to transform BL21 (DE3) bacteria, which were then induced with 0.1 mM isopropyl-1-thio-β-β-galactopyranoside and cultured for 4 h at 37 °C. Bacteria from a 1-liter culture were collected by centrifugation (6,000 × g, 30 min), washed with PBS (phosphate-buffered saline), and resuspended in 50 ml of 40 mM of 50 mM Tris-HCl, pH 8, 150 mM NaCl (lysis buffer) containing lysosyme (1 mg/ml final concentration), and protease inhibitors. Following incubation (1 h at 4 °C), protease inhibitors, DNase I (20 μg/ml final concentration), and 0.2% Triton X-100 (final concentration) were added, and the suspension was incubated for 1 h at 4 °C. The lysate was then subjected to sonication on ice (five pulses of 10 s each) and centrifuged (12,000 × g, 30 min). The supernatant was incubated with 1.5 ml of nickel-nitritotriacetic acid Superflow resin in the presence of 20 mM imidazole for 2 h at 4 °C. The resin was then poured into a column and washed successively with lysis buffer supplemented with 0.2% Triton X-100 and lysis buffer supplemented with 50 mM imidazole. Recombinant NAT1 was eluted with 300 mM imidazole. Purified NAT1 was reduced by incubation with 10 mM DTT for 10 min at 4 °C and then dialyzed against 25 mM Tris-HCl, pH 7.5, and 1 mM EDTA and stored at −80 °C. SDS-PAGE analysis was carried out at each stage of purification, and protein concentrations were determined with a standard Bradford assay.

Enzyme Assay—Detection of NAT1 activity in MCF7 cell extracts was performed as described previously (45) in a total volume of 100 μl. Cell extracts (50 μl) and p-aminobenzoic acid (200 μM) in assay buffer (20 mM Tris-HCl and 1 mM DTT, pH 7.5) were pre-incubated at 37 °C for 5 min. AcCoA (400 μM) was added to start the reaction, and the samples were incubated at 37 °C for different times (up to 30 min). The reaction was terminated with 100 μl of ice-cold aqueous 20% (w/v), and the proteins were pelleted by centrifugation for 5 min at 12,000 × g. 4-Dimethylaminobenzaldehyde (DMAB; 800 μl, 5% w/v in 9:1 acetonitrile/water) was added, and the absorbance was measured in 10-mm pathlength cuvettes at 450 nm (Uvikon Spectrophotometer). The amount of the remaining arylamine was determined from a standard curve. All assays were performed in triplicate in such conditions that the initial rates were linear. Enzyme activities were normalized according to the protein concentration of cell extracts and are expressed as percentages of control NAT1 activity (untreated MCF7 monolayers taken as 100%).

Recombinant NAT1 enzyme activity was determined spectrophotometrically at 410 nm using PNPA as the acetyl donor and PAS as a NAT1-specific arylamine substrate, as described by Mushtaq et al. (46). Briefly, samples (10–20 μl) were assayed in a reaction mixture containing 500 μM PAS (final concentration) in 25 mM Tris-HCl, pH 7.5, and 1 mM EDTA. Reactions were started by adding PNPA to a final concentration of 125 μM. All assays were carried out in a total volume of 1 ml such that the final concentration of NAT1 was always 15 nM. Reactions were incubated for 10 min at 37 °C and then quenched with SDS (1%). p-Nitrophenol, generated by the hydrolysis of PNPA by NAT1 in the presence of PAS, was quantified by measuring absorbance at 410 nm with an ELISA plate reader (Metertech). One enzyme unit was defined as the amount of enzyme giving an A_{410} of 0.5 per 10 min per milliliter. For the controls, we omitted the enzyme, PNPA, or PAS. Assays were performed in quadruplicate in conditions such that the initial rates were linear. Enzyme activities are expressed as percentages of control NAT1 activity (untreated enzyme taken as 100% activity). The total volume of the enzyme assay (1 ml) resulted in dilution of the various compounds used to a sufficient extent (in 50 or 1 in 100) to prevent interference with the enzyme activity.

Effects of Bolus Peroxynitrite and Peroxynitrite Generated by SIN1 on Recombinant NAT1 Activity in the Presence or Absence of Various Chemical Compounds—SIN1 is a chemical agent that mimics the generation of peroxynitrite occurring under physiological conditions by generating both superoxide and nitric oxide (47). These two molecules work together extremely rapidly (at a constant concentration limit), leading to the quantitative formation of peroxynitrite (37, 47). Thus, SIN1 is frequently used to generate peroxynitrite in chemical and biological experimental systems (47). We used SIN1 to generate peroxynitrite in all experiments except in the experiment that deter-
minded the rate constant for the reaction of peroxynitrite with NAT1. We assessed the effects on NAT1 activity of peroxynitrite and peroxynitrite released by SIN1 by incubating purified NAT1 (1.5 μM final concentration) with various concentrations of bolus peroxynitrite or SIN1 in 25 mM Tris-HCl, pH 7.5, and 1 mM EDTA (total volume of 10 μl) for 10 min at 37 °C. Mixtures were then assayed for NAT1 activity as described above.

We investigated the ability of reducing agents to protect NAT1 (1.5 μM final concentration) from the effects of peroxynitrite by carrying out SIN1 treatment in the presence or absence of various concentrations of DTT or GSH and then determining residual NAT1 activity. We assessed reactivation of the SIN1-treated enzyme by reducing agents as follows. NAT1 enzyme (1.5 μM final concentration) was first treated with SIN1 (250 μM final concentration) as described above. It was then incubated for 10 min at 37 °C with various concentrations of DTT or GSH in a total volume of 20 μl, and a NAT1 assay was carried out. Control assays in the conditions described above, with GSH or DTT only, gave 100% NAT1 activity.

We assessed the extent to which AcCoA and CoA protected against the SIN1-dependent inactivation of NAT1 as follows. NAT1 (1.5 μM final concentration) was first incubated with various concentrations of AcCoA or CoA (final concentrations of 100 μM to 5 mM) in 25 mM Tris-HCl, pH 7.5, and 1 mM EDTA (total volume of 10 μl) for 5 min at 37 °C. Samples were then incubated with SIN1 (250 μM final concentration) for 10 min at 37 °C in a total volume of 20 μl and assayed. Control assays carried out in the conditions described above, with AcCoA or CoA only, gave 100% NAT1 activity.

Kinetic Analysis of the Peroxynitrite-dependent Inactivation of NAT1—NAT1 (1.5 μM final concentration) was incubated with bolus peroxynitrite (final concentrations of 0–55 μM) at 37 °C in 25 mM Tris-HCl, pH 7.5, and 1 mM EDTA for 5 min at 37 °C and assayed for residual activity. We determined the second-order rate constant for the reaction of peroxynitrite and NAT1 (k_{inact}) by carrying out kinetic analysis as described by Radi et al. (48), taking into account the spontaneous degradation of peroxynitrite at pH 7.5 and 37 °C. Under these conditions, changes in peroxynitrite (PN) and NAT1 concentrations as a function of time are expressed as shown in Equations 1 and 2,

\[
\frac{d[PN]}{dt} = -k_{inact}[NAT1][PN] - k_{dec}[PN] \tag{1}
\]

\[
\frac{d[NAT1]}{dt} = -k_{inact}[NAT1][PN] + k_{inact} \tag{2}
\]

where [NAT1] is the concentration of the enzyme, [PN] the concentration in peroxynitrite, k_{inact} the second-order rate constant for the inactivation of NAT1 by peroxynitrite, and k_{dec}, the first-order rate constant for the decomposition of peroxynitrite. In our conditions (37 °C, pH 7.5), the value of k_{dec} has been shown to be 0.9 s⁻¹ (48). The value of k_{inact} was calculated by fitting an equation derived by Radi et al. (48) to the data, as shown in Equation 3.

\[
\ln \frac{[NAT1]}{[NAT1]_{0}} = k_{dec}[PN] \text{ where } k_{obs} = \frac{k_{inact}}{k_{inact} + k_{dec}} \tag{3}
\]

and where [NAT1] and [PN], are the initial concentrations of NAT1 and peroxynitrite, respectively, and [NAT1]₀ is the final concentration of active NAT1 after a reaction with a given concentration of peroxynitrite. Finally, Equation 3 predicts that a plot of ln of fractional activity of NAT1 versus the initial concentration of peroxynitrite should be linear. The value of k_{obs} is deduced from the slope (bᵣ). Kinetic data were plotted and fitted using KaleidaGraph version 3.5 (Abelbeck Software).

Fluorescein-conjugated Iodoacetamide Labeling of Proteins—Purified NAT1 (1.5 μM final concentration) was incubated with or without (control) various concentrations of SIN1 (from 50 to 500 μM final concentration) in 25 mM Tris-HCl, pH 7.5, and 1 mM EDTA for 10 min at 37 °C. Samples were then incubated with fluorescein-conjugated iodoacetamide (20 μM final concentration) for 10 min at 37 °C and subjected to SDS-PAGE under reducing conditions and Western blotting with anti-fluorescein Fab’ fragments conjugated to peroxidase.

Statistical Analysis—Data are expressed as means ± S.D. of three independent experiments performed in quadruplicate. The statistical significance of differences between means was evaluated using Student’s t test. The level of significance was set at p = 0.05.

Protein Determination, SDS-PAGE, and Western Blotting—Protein concentrations were determined by the Bradford assay (BioRad). Samples for gel electrophoresis were mixed with reducing or non-reducing 4× SDS sample buffer and separated by SDS-PAGE. Gels were stained with Coomassie Brilliant Blue R-250. For Western blotting after separation by SDS-PAGE, proteins were electrotransferred to a nitrocellulose membrane. The membrane was blocked by incubation with Tris-buffered saline/Tween 20 (TBS) supplemented with 5% nonfat milk powder for 1 h. Anti-fluorescein Fab’ fragments conjugated to horseradish peroxidase (1:100000) in TBS were added. The membrane was then incubated for 1 h and washed with TBS. The Supersignal reagent (Pierce) was used for detection.
described above were also carried out with SIN1 instead of peroxynitrite. Decomposed SIN1 had no effect on recombinant NAT1 activity.

The sensitivity of human NAT1 to peroxynitrite-dependent inactivation was due to SIN1-generated peroxynitrite. Decomposed SIN1 had no effect on recombinant NAT1 activity.

Overall, these results suggest that human NAT1 is inactivated continuously, led to a weaker but significant inactivation of cellular NAT1 (Fig. 2B, top). This weaker effect is likely due to the fact that that SIN1 generates lower levels of peroxynitrite (28, 51) than the parent peroxynitrite donor (lower by a factor of at least 4; Ref. 51). As demonstrated by comparative expression level analyses of cellular NAT1 by immunoprecipitation and Western blotting (Fig. 2, A and B, bottom), inactivation obtained with peroxynitrite and SIN1 was not due to proteolysis or changes in NAT1 protein expression induced by the exposures to these compounds. Decomposed SIN1 (obtained by allowing decomposition at room temperature in the dark for 48 h), which does not generate peroxynitrite, had no effect on cellular NAT1 (Fig. 2B, top and bottom), indicating that enzyme inactivation was due to SIN1-generated peroxynitrite. Overall, these results suggest that human NAT1 is inactivated in MCF7 cells by concentrations of peroxynitrite that are physiologically relevant (49). To gain further insights into the mechanisms by which peroxynitrite inactivates cellular and recombinant NAT1, further kinetic and biochemical analyses were carried out on the recombinant NAT1 enzyme as described below.

Peroxynitrite and SIN1-generated Peroxynitrite Inactivates NAT1 in Cultured Breast Cancer Cells—We assessed the sensitivity of human NAT1 to peroxynitrite-dependent inactivation by exposing cultured human breast cancer cells to physiological concentrations of synthetic peroxynitrite. In addition, we also mimicked physiological peroxynitrite generation by exposing these cells to SIN1, a compound that releases both superoxide and nitric oxide at a constant rate, leading to the quantitative formation of peroxynitrite by means of a reaction that is almost diffusion-limited (32, 37, 38). SIN1 has been shown to attack many biological targets in the same manner as authentic peroxynitrite and, thus, has been widely used as a source of peroxynitrite in studies using purified proteins and cultured cells (47). Physiological concentrations of peroxynitrite in vivo have been estimated to be ~50 μM (49), although 500 μM concentrations have been found within phagolysosomes of activated macrophages (50).

As shown in Fig. 2, exposure of MCF7 cells to both peroxynitrite (Fig. 2A, top) and SIN1-released peroxynitrite (Fig. 2B, bottom) caused significant concentration-dependent inactivation of the endogenous NAT1 enzyme. At 50 μM, ~40% of cellular NAT1 was inactivated by a 10-min exposure to peroxynitrite (Fig. 2A, top). Complete inactivation of cellular NAT1 was obtained with 250 μM peroxynitrite (Fig. 2A, top). Exposure for 30 min to SIN1, which releases peroxynitrite continuously, led to a weaker but significant inactivation of cellular NAT1 (Fig. 2B, top). This weaker effect is likely due to the fact that that SIN1 generates lower levels of peroxynitrite (28, 51) than the parent peroxynitrite donor (lower by a factor of at least 4; Ref. 51). As demonstrated by comparative expression level analyses of cellular NAT1 by immunoprecipitation and Western blotting (Fig. 2, A and B, bottom), inactivation obtained with peroxynitrite and SIN1 was not due to proteolysis or changes in NAT1 protein expression induced by the exposures to these compounds. Decomposed SIN1 (obtained by allowing decomposition at room temperature in the dark for 48 h), which does not generate peroxynitrite, had no effect on cellular NAT1 (Fig. 2B, top and bottom), indicating that enzyme inactivation was due to SIN1-generated peroxynitrite. Overall, these results suggest that human NAT1 is inactivated in MCF7 cells by concentrations of peroxynitrite that are physiologically relevant (49). To gain further insights into the mechanisms by which peroxynitrite inactivates cellular and recombinant NAT1, further kinetic and biochemical analyses were carried out on the recombinant NAT1 enzyme as described below.

RESULTS

Peroxynitrite and SIN1-generated Peroxynitrite Inactivates NAT1 in Cultured Breast Cancer Cells—We assessed the sensitivity of human NAT1 to peroxynitrite-dependent inactivation by exposing cultured human breast cancer cells to physiological concentrations of synthetic peroxynitrite. In addition, we also mimicked physiological peroxynitrite generation by exposing these cells to SIN1, a compound that releases both superoxide and nitric oxide at a constant rate, leading to the quantitative formation of peroxynitrite by means of a reaction that is almost diffusion-limited (32, 37, 38). SIN1 has been shown to attack many biological targets in the same manner as authentic peroxynitrite and, thus, has been widely used as a source of peroxynitrite in studies using purified proteins and cultured cells (47). Physiological concentrations of peroxynitrite in vivo have been estimated to be ~50 μM (49), although 500 μM concentrations have been found within phagolysosomes of activated macrophages (50).

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Recombinant NAT1 Is Rapidly Inactivated by Peroxynitrite—In parallel with the MCF7 cell study, recombinant purified NAT1 was exposed to a physiological concentration of peroxynitrite or to SIN1 (Fig. 3). As obtained for cellular NAT1 (Figs. 3A and 1B, top), recombinant NAT1 was inactivated by physiological concentrations of peroxynitrite (Fig. 3A) and by SIN1 in a dose-dependent manner (Fig. 3B). We carried out kinetic analysis as described by Radi et al. (48) and calculated the rate constants for reactions involving peroxynitrite. Our data gave a linear relationship, according to Equation 3 (see “Materials and Methods”), with a second-order rate constant for the peroxynitrite-mediated inactivation of NAT1 (k_inact) of $5 \times 10^4 \text{m}^{-1}\text{s}^{-1}$ (Fig. 4). This value is similar to the $k_{\text{inact}}$ reported for other peroxynitrite-inactivated enzymes, such as tryptophan hydroxylase ($k_{\text{inact}} = 3.4 \times 10^4 \text{m}^{-1}\text{s}^{-1}$) (35), and indicates that the inactivation of human NAT1 by peroxynitrite occurs rapidly.

Modification of the Sulfhydryl Groups of NAT1 by Peroxynitrite Released by SIN1—Peroxynitrite is a powerful sulfhydryl oxidant (52). However, it can also modify the tyrosine residues of proteins to yield 3-nitrotirosine, and this modification may lead to the irreversible inactivation of enzymes (39, 49, 52). Treatment of human NAT1 with N-acetylimidazole (20 mM), a tyrosine-modifying agent, did not affect the activity of the enzyme (data not shown), confirming that the tyrosine residues of NAT1 are not involved in its catalytic activity (11). We therefore investigated whether peroxynitrite modified NAT1 cysteine residues. We incubated NAT1 with SIN1 in the concentration range shown to inactivate the enzyme and treated it with fluorescein-conjugated iodoacetamide, which binds free, unmodified sulfhydryl groups, as described previously (53). The prior incubation of NAT1 with various concentrations of SIN1 (50–500 μM) resulted in a higher level of cysteine residue modification, as indicated by the disappearance of fluorescein-iodoacetamide labeling (Fig. 5). These results provide clear evidence that NAT1 cysteine residues are modified by peroxynitrite.

Peroxy nitrite-mediated Inactivation of NAT1 Is Irreversible—We assessed whether peroxynitrite-dependent inactivation of NAT1 was irreversible. To this end, NAT1 was first incubated with SIN1. The resulting inactivated NAT1 was then incubated with reduced GSH or DTT at final concentrations of 1 or 5 mM for 10 min at 37 °C. These reducing agents did not significantly reverse SIN1-induced enzyme inactivation (Fig. 6A). A weak reactivation effect (~30% control activity) was obtained with the highest concentration of DTT (5 mM final concentration). Thus, as demonstrated for other peroxynitrite-sensitive enzymes (28, 35, 50, 54), peroxynitrite irreversibly inactivated NAT1. Our data also suggest that peroxynitrite oxidized the sulfhydryl groups of NAT1 sulfenic acid beyond the sulenic acid, as shown in other cases (35).

Protective Effect of Thiol Reducing Agents against the Peroxynitrite-dependent Inactivation of NAT1—We assessed the ability of reducing agents to prevent the SIN1-induced inactivation of NAT1 (Fig. 6B). Incubation of the enzyme with SIN1 in the presence of high concentrations of reducing agents (5 mM GSH or 5 mM DTT) protected NAT1 only partially against inactivation. DTT protected against inactivation more efficiently than GSH, as reported for other enzymes (35). At a final GSH concentration of 5 mM (corresponding to 20-fold excess of GSH over SIN1), ~60% residual NAT1 activity was obtained. Full protection of NAT1 against SIN1-induced inactivation was obtained only with very high final concentrations of GSH or DTT exceeding 10 mM (data not shown). These results suggest that GSH provides significant protection against NAT1 inactivation only at high levels. However, these data also suggest that peroxynitrite reacts more rapidly with NAT1 than with GSH, which is consistent with the $k_{\text{inact}}$ reported here ($k_{\text{inact}} = 5 \times 10^4 \text{m}^{-1}\text{s}^{-1}$) being higher than the rate of reaction of peroxynitrite with GSH ($k = 10^3 \text{m}^{-1}\text{s}^{-1}$) (32). In addition, these results are in agreement with the cellular data described above (Fig. 2, A and B), which show that the reducing intracellular environment does not protect endogenous NAT1 sufficiently from peroxynitrite-dependent inactivation.

Identification of the Site Modified during SIN1-dependent Inactivation of NAT1—Our results strongly suggested that the inactivation of NAT1 by peroxynitrite was due to irreversible modification of the cysteine residues of the enzyme. The catalytic activity of NAT enzymes depends on a reactive cysteine residue present in a cysteine protease-like triad. This cysteine residue is known to form a covalent acetylcycteinyli enzyme with the acetyl group of AcCoA, the physiological acetyl donor substrate of NATs (11). We therefore investigated whether AcCoA and CoA (a product resulting from the hydrolysis of AcCoA, which does not form an acetyl enzyme) protected human NAT1 against peroxynitrite-dependent inactivation. To this end, a substrate protection assay using AcCoA and CoA as control was carried out as described elsewhere (25, 55, 56). This method has been used to show that the acetyl moiety of AcCoA is specifically transferred to the unique catalytic cysteine residue of NATs, leading to the formation of an acetyl-enzyme intermediate (24, 55). As shown in Table I, the incubation of NAT1 with AcCoA significantly protected the enzyme against inactivation by a SIN1-generated peroxy nitrite. In contrast, CoA, which cannot form an acetyl-cysteine with the fundamen-
dependent inactivation of NAT1 is irreversible, as catalytic due to specific oxidative modification of the catalytic cysteine residue of the enzyme, provided no protective action. Thus, the peroxynitrite-dependent inactivation of human NAT1 is due to irreversible oxidation of the catalytic cysteine residue of the enzyme.

**DISCUSSION**

The role of NATs in the detoxification/activation of various carcinogens has led to many studies focusing on the relationship between the allele frequencies of NAT1 and/or NAT2 and cancer risk (57). Interestingly, NAT1 expression has been linked to carcinogenesis and tumor progression. Indeed, the region of chromosome 8 on which the NAT1 gene is located is commonly deleted in certain neoplastic cells (6), suggesting that the absence or catalytic inactivation of NAT1 may contribute to carcinogenesis and/or tumor progression (6). Recent data showed that non-genetic factors such as the substrate-dependent inhibition of NAT1 activity may contribute to low NAT1 activity in vivo (8, 9). In addition, NAT1 has also been shown to be inactivated by oxidants in vitro (25, 56).

Peroxynitrite is detected in different tissues where it has been implicated in physiological and pathophysiological processes such as vasorelaxation, apoptosis, or inflammation (30–33). In addition, peroxynitrite plays a major role in carcinogenesis and tumor progression leading to peroxynitrite-dependent modification of proteins, in particular in patients with colorectal and breast carcinoma (43, 44, 58). Moreover, different enzymes such as cytochromes P450 (38, 39) and glutathione S-transferase (40) have been shown to be inhibited by peroxynitrite. In this work, we studied whether exposure of human breast cancer cells to physiological concentrations of peroxynitrite and a peroxynitrite-generating compound (SIN1) led to the inactivation of the cellular NAT1 enzyme. Human breast cancer cells are known to express the NAT1 but not the NAT2 enzyme (17). We clearly showed here that endogenous NAT1 expressed by MCF7 cells is inactivated by a short exposure of these cells to physiological concentrations of peroxynitrite (close to 50 μM; Ref. 49), but concentrations of up to 500 μM have also been reported Ref. 50. The rate constant for the inactivation of recombinant NAT1 by peroxynitrite was determined to be $5 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$, indicating that the reaction between peroxynitrite and the enzyme is extremely fast. This rate constant is similar to that determined for other peroxynitrite-regulated enzymes such as tryptophan hydroxylase (35). More importantly, substrate protection experiments clearly show that the peroxynitrite-dependent inactivation of NAT1 is due to specific oxidative modification of the catalytic cysteine residue of the enzyme. In addition, as reported for other enzymes (32, 34, 37), we demonstrate here that the peroxynitrite-dependent inactivation of NAT1 is irreversible, as catalytic activity was not fully restored by reducing agents present in excess. This indicates that the catalytic cysteine residue was oxidized to sulfinic and/or sulfonic acids (48). Thus, our results suggest that NAT1, a phase II XME, may be a physiological target of transient or chronically generated peroxynitrite. Other phase I and phase II XMEs, such as cytochromes P450 (38, 39) and glutathione S-transferases (40), have also been reported to be irreversibly inactivated by peroxynitrite.

Our results show that, even in the presence of a high concentration of GSH (5 mM), peroxynitrite (at a molar concentration at least 20 times smaller than that of GSH) was still able to react and inactivate NAT1 (Fig. 6B), suggesting that peroxynitrite reacts more rapidly with the catalytic cysteine of NAT1 than the thiol group of GSH. Consistent with this, peroxynitrite interacts with the catalytic cysteine residue of NAT1 with a rate constant ($k_{3/2}$) of $5 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$, whereas it interacts with GSH —50 times slower ($k = 10^3 \text{ M}^{-1}\text{s}^{-1}$) (32). This may explain why the reducing intracellular environment of MCF7 cells did not protect endogenous NAT1 sufficiently from peroxynitrite-dependent inactivation (Fig. 2, A and B). GSH is known to be the major determinant of cellular redox potential and is generally present at concentrations of ~1–2 mM, although concentrations of up to 10 mM have been reported (59). Our results suggest that a high concentration of GSH (>5 mM) can provide partial protection (Fig. 6B), suggesting that high GSH levels in cells could modulate the effect of peroxynitrite on NAT1 activity. Interestingly, it is known that oxidative stress-dependent GSH depletion may affect the bio-transformation activity of the phase II XME, glutathione-S-transferase (4).

Non-genetic factors that affect NAT1 activity and that may account for the lack of a clear association between NAT1 phenotypes and the risk of certain cancers (5) have been seen. Reactive nitrogen species and, in particular, peroxynitrite have been shown to play a major role in carcinogenesis, affecting diverse processes including inflammation, proliferation, apoptosis, tumorigenesis, and metastasis (30, 32, 41, 42). Interestingly, recent data have expanded the concept that the inflammation and generation of peroxynitrite are critical components of cancer progression (60). In addition, inflammatory response is increasingly recognized as a source of variability in the pharmacokinetics and pharmacodynamics of cancer therapy (61). Our results strongly suggest that peroxynitrite and reactive nitrogen species in general may inhibit NAT1 activity in vivo. Moreover, our results, in addition to the data obtained for other XMEs, provide possible mechanisms by which the increased generation of peroxynitrite may favor carcinogenesis by inactivating key cellular defense enzymes.

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