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The Xenobiotic-Metabolizing Enzymes Arylamine *N*-Acetyltransferases in Human Lens Epithelial Cells: Inactivation by Cellular Oxidants and UVB-Induced Oxidative Stress

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ABSTRACT

The human arylamine *N*-acetyltransferases NAT1 and NAT2 are important xenobiotic-metabolizing enzymes involved in the detoxification and metabolic activation of numerous drugs and chemicals. NAT activity depends on genetic polymorphisms and on environmental factors. It has been shown that low NAT-acetylation activity could increase the risk of age-dependent cataract, suggesting that NAT detoxification function may be important for lens cells homeostasis. We report here that the NAT acetylation pathway may occur in human lens epithelial (HLE) cells. Functional NAT1 enzyme was readily detected in HLE cells by reverse transcription-polymerase chain reaction, Western blotting, and enzyme activity assays. NAT2 mRNA and enzymic activity were also detected. We investigated whether oxidants, known to be produced in HLE cells during oxidative stresses and involved in age-dependent cataract formation, decreased endogenous NAT1 and NAT2 activity. The exposure of HLE cells to peroxynitrite led to the dose-dependent irreversible inactivation of both NAT isoforms. Exposing HLE cells to continuously generated H_2O_2 gave a dose-dependent inactivation of NAT1 and NAT2, reversible on addition of high concentrations of reducing agents. UVB irradiation also induced the reversible dose-dependent inactivation of endogenous NAT1 and NAT2, reversible on addition of reducing agents. Thus, our data suggest that functional NAT1 and NAT2 are present in HLE cells and may be impaired by oxidants produced during oxidative and photooxidative stresses. Oxidative-dependent inhibition of NATs in these cells may increase exposure of lens to the harmful effects of toxic chemicals that could contribute to cataractogenesis over time.

Arylamine *N*-acetyltransferases (NATs; EC2.3.1.5) are XMEs that catalyze the acetyl-coenzyme A (AcCoA)-dependent *N*-acetylation of many arylamine and *O*-acetylation of hydroxylated heterocyclic amines (Pompeo et al., 2002). NAT-dependent acetylation is a major biotransformation pathway for various drugs and environmental xenobiotics (Hein et al., 2000). In humans, two functional NAT isoforms, NAT1 and NAT2, are encoded by two polymorphic loci (Matas et al., 1997). Interindividual genetic variations lead to differences in NAT activity and are potential sources of toxicological/pathological susceptibility (Hein et al., 2000). Substrate-dependent down-regulation (Butcher et al., 2000, 2004) and oxidative-dependent inactivation (Atmane et al., 2003; Dairou et al., 2003, 2004) may also regulate NAT activity. Therefore, it seems that the overall NAT activity in cells depends on both genetic and environmental factors. Despite having very similar amino acid sequences (81% identical), human NATs differ in terms of substrate specificity profiles (Pompeo et al., 2002) and tissue expression patterns (Sim et al., 2000). NAT1 mRNA and protein are present in a wide range of tissues (Sim et al., 2000; Rodrigues-Lima et al., 2003). NAT2 mRNA and protein have been detected in liver (Deguchi, 1992) and colon epithelium (Ilett et al., 1994). NAT2 mRNA has been detected in a wider range of tissues, suggesting that NAT2 enzyme may be present in many more

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ABBREVIATIONS: NAT, arylamine *N*-acetyltransferase; XME, xenobiotic-metabolizing enzyme; AcCoA, acetyl-coenzyme A; SIN1, 3-morpholinosydnonimine *N*-ethylcarbamide; PAS, *p*-aminosalicylic acid; SMZ, sulfamethazine; DTT, dithiothreitol; GSH, reduced glutathione; MEM, minimal essential medium; PBS, phosphated-buffered saline; GOX, glucose oxidase; RT-PCR, reverse transcription-polymerase chain reaction; PCR, polymerase chain reaction; bp, base pair(s); DMAB, 4-dimethylaminobenzaldehyde; PAGE, polyacrylamide gel electrophoresis.

tissues (Rodrigues-Lima et al., 2003). Crystal structures of bacterial NATs (Sinclair et al., 2000; Sandy et al., 2002; Westwood et al., 2005) and homology modeling of human NATs (Rodrigues-Lima et al., 2001; Rodrigues-Lima and Dupret, 2002) have revealed the presence of a conserved cysteine protease-like catalytic triad (Cys-His-Asp). This active site cysteine residue is critical for NAT activity (Dupret and Grant, 1992), and its oxidative modification by oxidants leads to the inactivation of NAT enzymes (Mattano et al., 1989; Atmane et al., 2003; Dairou et al., 2004).

The human lens has a single layer of metabolically active epithelial cells. This layer is essential for maintenance of the homeostasis and transparency of the entire lens (Spector, 1991). The exposure of human lens epithelial (HLE) cells to endogenous or environmental stress factors, such as oxidative stress, ultraviolet (UV) radiation and toxic chemical agents may lead to pathological eye conditions (Spector, 1991; Davies and Truscott, 2001; Dudek et al., 2001). Oxidative damage to the macromolecules of HLE cells caused by increases in the levels of cellular oxidants by UV-dependent photooxidative processes, exposure to toxic chemicals, or failure of antioxidant defense systems is causally related to age-dependent cataract formation (Halliwell and Gutteridge, 1999; Davies and Truscott, 2001). Peroxynitrite and H_2O_2 are thought to be the major oxidants to which HLE cells are acutely or chronically exposed (Halliwell and Gutteridge, 1999; Paron et al., 2004; Thiagarajan et al., 2004). Increases in the levels of these oxidants in HLE cells are known to cause oxidative-dependent inactivation of key enzymes, including proteasome, catalase, and glucose-6-phosphate dehydrogenase (Reddy et al., 2001; Hosler et al., 2003; Rogers et al., 2004; Thiagarajan et al., 2004). Exposure to UVB radiation, a major risk factor for cataract formation, can also alter HLE cell proteins and inactivate lenticular enzymes (Davies and Truscott, 2001; Reddy et al., 2001). NAT-dependent detoxification pathway has been suggested to play a protective role in lens cells. Indeed, NAT2 slow acetylator individuals have been shown to be at greater risk of developing age-dependent cataract than fast acetylators, which suggest that exogenous chemical factors detoxified by NATs may be etiological agents for cataract formation (Meyer et al., 2003). Therefore, in addition to genetic polymorphisms, oxidative-dependent inactivation of NATs may have important consequences for xenobiotic detoxification in HLE cells.

We show here that functional NAT1 is present in HLE cells, the enzyme being readily detected at the mRNA, catalytic, and protein levels. NAT2 mRNA and enzymic activity were also detected, but protein levels were below the detection thresholds for Western blotting using our anti-NAT2 antibody. Because NATs are inactivated in vitro by oxidative modifications of their catalytic cysteine residue (Rodrigues-Lima and Dupret, 2004), we tested whether oxidants known to be produced acutely or chronically at elevated levels in HLE cells during oxidative stresses (Halliwell and Gutteridge, 1999; Thiagarajan et al., 2004) could affect endogenous NAT1 and NAT2 activity. We found that peroxynitrite and H_2O_2 inhibited endogenous NAT1 activity. The exposure of HLE monolayers to physiologically relevant doses of UVB radiation led to the oxidative-dependent inactivation of cellular NAT1 activity, reversible on the addition of reducing agents. A similar pattern of inactivation of endogenous NAT2 was found in HLE cells exposed to peroxynitrite, H_2O_2, or UVB.

Our data show that functional NAT enzymes are present in HLE cells. Moreover, elevated levels of oxidants in HLE cells during oxidative and/or photooxidative stress may decrease endogenous NAT activity. This oxidative-dependent impairment of NAT detoxification activity in HLE cells may increase the exposure of lens cells to harmful chemicals that could contribute to age-related cataract formation over time.

Materials and Methods

Materials. Peroxynitrite and 3-morpholinosydnonimine *N*-ethylcarbamide hydrochloride (SIN1) were obtained from Calbiochem-Novabiochem (San Diego, CA). *p*-Aminosalicylic acid (PAS), sulfamethazine (SMZ), AcCoA, dithiothreitol (DTT), reduced glutathione (GSH), glucose oxidase (type II from *Aspergillus niger*, 15,500 units/g), bovine catalase (5200 units/mg), β-D-glucose, and monoclonal anti-human actin antibody were obtained from Sigma-Aldrich (St. Louis, MO). The Bradford protein assay kit was supplied by Bio-Rad (Hercules, CA). All other reagents were purchased from Sigma-Aldrich or Eurobio (Les Ullis, France). Polyclonal antibodies against human NAT1 and human NAT2 were kindly provided by Prof. E. Sim (University of Oxford, Oxford, UK) and have been described previously (Stanley et al., 1996; Rodrigues-Lima et al., 2003). HLEB3 cells were obtained from American Type Culture Collection (Manassas, VA) and are derived from human infant lens epithelium fragments. FHL124 cells were kindly provided by Prof. J. Reddan (Oakland University, Rochester, MI) and are derived from human adult lens capsule-epithelial explants.

Cell Culture, Peroxynitrite/SIN1, Hydrogen Peroxide, and UV Treatments. HLEB3 and FLH124 cells (human lens epithelial cell lines) were cultured as monolayers in modified Eagle's minimum essential medium (MEM; American Type Culture Collection) supplemented with 20% (v/v) fetal bovine serum and penicillin/streptomycin in 100-mm Petri dishes at 37°C. Primary cultures of rabbit lens epithelial cells were obtained and cultured as described previously (Couder et al., 1999).

HLEB3 cells are widely used as a model for in vivo studies of HLE cell physiology, eye-related toxicology, and cataracts (Andley et al., 1994; Hosler et al., 2003). We used these cells to analyze the effect of cellular oxidants and UVB radiation on the activity of the endogenous NAT1 and NAT2 enzymes. All treatments were performed at least in triplicate.

At ~90% confluence, HLEB3 cell monolayers were washed with PBS. Cell monolayers were exposed to various concentrations of peroxynitrite (100–400 μM) or SIN1 (500 μM–2 mM) in 10 ml of serum-free PBS or serum-free MEM, with incubation for 10 min (peroxynitrite) or 30 min (SIN1) at 37°C. Decomposed SIN1 (obtained by decomposition at room temperature in the dark for 24 h), and PBS or MEM alone were used as controls. We investigated the effects of H_2O_2 by treating cell monolayers with H_2O_2 generated continuously (~5 μM/min) in situ by the glucose/glucose oxidase system (GOX), as described previously (Ravid et al., 2002; Atmane et al., 2003). In brief, monolayers were incubated with 1.5 U/ml glucose oxidase and 5 mM glucose in PBS at 37°C for various lengths of time. As a control, monolayers were incubated with 300 U/ml catalase. Glucose oxidase, glucose, and catalase had no effect on NAT1 activity when tested independently (Atmane et al., 2003). We assessed the effect of UVB irradiation by subjecting HLEB3 monolayers in PBS or MEM to physiologically relevant levels of UVB (100, 200, and 400 J/m^2; 3UV transilluminator; OSI, Elancourt, France; maximum emission of UVB lamps at 302 nm) as described previously (Rogers et al., 2004; Shin et al., 2004) and incubating them at 37°C for 15 min.

After treatment, monolayers were washed with PBS, scraped into 800 μl of lysis buffer (20 mM Tris-HCl, pH 7.5, 0.2% Triton X-100, 1 mM EDTA, and protease inhibitors) and centrifuged for 30 min at

100,000*g*. Supernatants (total cell extract) were used to determine protein concentration by means of the Bradford assay (Bio-Rad). Lysis buffer was added to cell extracts to give final extracts of equal protein concentration. Normalization of cell extracts was also checked on Western blots with anti-actin antibody.

We assessed the ability of GSH and DTT to reactivate endogenous inactivated NAT1 and NAT2 enzymes. We incubated treated extracts with GSH or DTT (10 mM final concentration) for 10 min at 4°C in a total volume of 400 μl before enzyme assays.

RNA Isolation and Reverse Transcription-Polymerase Chain Reaction. Total RNA was isolated from HLE cell monolayers (HLEB3 and FHL124 cell lines) using TRIzol (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. First-strand cDNA was synthesized from 6 μg of total RNA at 70°C, in a final volume of 20 μl, using the *Carboxydothermus hydrogenoformans* polymerase reverse transcription-polymerase chain reaction (RT-PCR) kit (Roche Diagnostics, Indianapolis, IN) and reverse primers specific for human NAT1 or NAT2 (see below). For PCR analysis, 10 μl of cDNA was used as a template in a 50-μl amplification mixture containing *Taq*DNA polymerase (Sigma-Aldrich). The NAT primers used for PCR were for human NAT1 (specific 367-bp fragment), sense 5′-tagaagacagcaaatacc-3′, antisense 3′-gatgacaaatagacaagatt-5′; for human NAT2 (specific 412-bp fragment), sense 5′-tgccaaagaagaaacacc-3′, antisense 3′-gagaggatatctgatagcacata-5′. The PCR conditions were as follows: 35 cycles of 1-min denaturation at 94°C, 1-min annealing at 47°C, and 1-min extension at 72°C. The absence of genomic DNA contamination was confirmed by means of negative controls containing no reverse transcriptase. The PCR products were separated by gel electrophoresis in ethidium bromide-stained 1.8% agarose gels.

Enzyme Assay. The activities of NAT1 and NAT2 were detected in HLE cell extracts as described previously (Sinclair et al., 1998), in a total volume of 100 μl. Cell extracts (50 μl) and arylamine (200 μM PAS or SMZ) in assay buffer (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, with or without reducing agents) were incubated at 37°C for 5 min. PAS was used as the NAT1-specific arylamine substrate, and SMZ was used as the NAT2-specific arylamine substrate. We added 400 μM AcCoA to start the reaction, and the samples were incubated at 37°C for various lengths of time (up to 30 min). The reaction was quenched by adding 100 μl of ice-cold aqueous trichloroacetic acid [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 absorbance at 405 nm was measured in cuvettes with a 10-mm pathlength (spectrophotometer from Uvikon, St Quentin en Yvelines, France). The amount of residual arylamine was determined from a standard curve. All assays were performed in quadruplicate, in conditions giving a linear initial rate. The enzyme activities of treated cell extracts were normalized based on protein concentration and expressed as percentages of control NAT1 activity (control taken as 100% activity).

Detection of Endogenous NAT1 and NAT2 Proteins in Human Lens Epithelial Cell Extracts. We determined the amount of NAT1 and NAT2 in HLEB3 and FHL124 cell extracts by immunoprecipitating NAT1 or NAT2 overnight at 4°C with a purified anti-NAT1 or anti-NAT2 antibody (1 μg of IgG fraction) in a total volume of 300 μl. Protein A-agarose was added, and the mixture was incubated for 2 h at 4°C. Beads were washed twice with PBS. Bound enzymes were eluted by boiling in nonreducing SDS sample buffer and subjected to SDS-PAGE and Western blotting with the anti-NAT1 or the anti-NAT2 antibody.

Statistical Analysis. Data are expressed as the means ± S.D. of three independent experiments, with quadruplicate assays performed for each experiment. We assessed the statistical significance of differences between means with 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 (Bio-Rad). Samples for gel electrophoresis were mixed with reducing or nonreducing 4× SDS sample buffer and separated by SDS-PAGE. Gels were stained with Coomassie Brilliant Blue R-250. For Western blotting, proteins were separated by SDS-PAGE and electrotransferred to a nitrocellulose membrane. The membrane was blocked by incubation with Tris-buffered saline/Tween 20 supplemented with 5% nonfat milk powder for 1 h. Primary antibody (anti-NAT1 or anti-NAT2 at 1:1000) was added and the membrane incubated for a further 1 h 30 min in Tris-buffered saline/Tween 20 (1% milk powder) and washed. Secondary conjugated antibody (anti-rabbit antibody at 1:100,000) was added and the membrane incubated for 1 h. The membrane was then washed and the Supersignal reagent (Pierce Chemical, Rockford, IL) was used for detection.

Results

Presence of NAT Enzymes in Human Lens Epithelial Cells. We first investigated the presence of human NAT1 and NAT2 in HLEB3 and FHL124 HLE cells by RT-PCR, using specific primers (Kawakubo et al., 2000; Rodrigues-Lima et al., 2003), yielding PCR products of different sizes (367 bp for NAT1 and 412 bp for NAT2) (Fig. 1A). Amplicons

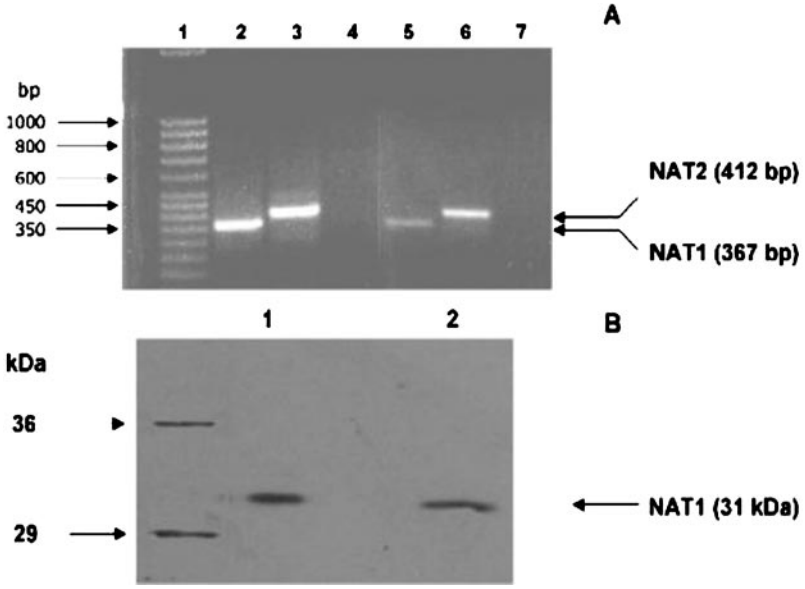

Fig. 1. Endogenous production of NATs in HLEB3 and FHL124 human lens epithelial cells. A, sequence-specific primers for human NAT1 and NAT2 were used for RT-PCR with total RNA from HLEB3 and FHL124 human lens epithelial cells. RT-PCR products were separated by electrophoresis in 1.8% agarose gels and were detected by ethidium bromide staining. Specific fragments, 367 bp (NAT1) and 412 bp (NAT2), were obtained with HLEB3 (lanes 2 and 3) and FHL124 (lanes 5 and 6). The absence of genomic DNA contamination was confirmed with negative controls in which no reverse transcriptase was added for the NAT1 RT experiment (HLEB3, lane 4; FHL124, lane 7). B, NAT1 and NAT2 were immunoprecipitated from equal amounts of HLEB3 (lane 2) and FHL124 (lane 3) protein extracts and were detected by Western blotting with specific antibodies (Stanley et al., 1996; Rodrigues-Lima et al., 2003). No NAT2 protein was detected with the specific anti-NAT2 antibody (data not shown).

corresponding to NAT1 and NAT2 (at 367 bp and 412 bp for NAT1 and NAT2, respectively) were detected in HLEB3 (lanes 2 and 3) and FHL124 (lane 5 and 6) cells. The absence of genomic DNA contamination was confirmed by negative controls in which no reverse transcriptase was added (lanes 4 and 7, for HLEB3 and FHL124, respectively). Thus, mRNAs encoding NAT1 and NAT2 are present in HLE cells. We investigated whether the NAT1 and NAT2 proteins were present in HLE cells by carrying out immunoprecipitation and Western blotting experiments with specific anti-NAT1 and anti-NAT2 antibodies described in previous studies (Stanley et al., 1996; Rodrigues-Lima et al., 2003). NAT1 protein was immunoprecipitated from HLEB3 (Fig. 1B, lane 1) and FHL124 (Fig. 1B, lane 2) cell extracts. We also tried to detect NAT2 protein in these samples. However, NAT2 protein could not be detected at the sensitivity achieved in these experiments (data not shown), probably because the anti-NAT2 antibody was derived from an antiserum with a lower titer than the NAT1 antiserum (Stanley et al., 1996). We also investigated whether active NAT1 and NAT2 enzymes were present in the HLE cell extracts, using PAS (NAT1 substrate) and SMZ (NAT2 substrate) in AcCoA-dependent acetylation assays (Table 1) as described by Hickman et al. (1998). NAT1 activity was readily detected in HLEB3 (5.9 ± 0.4 nmol/mg/min) and FHL124 (2.34 ± 0.9 nmol/mg/min) cell extracts. Reproducible, but lower levels of NAT2 activity (0.8 ± 0.05 nmol/mg/min in HLEB3 cells and 0.6 ± 0.02 nmol/mg/min in FHL124 cells) were also detected. Assays with extracts from primary cultures of rabbit lens epithelial cells gave overall similar results with NAT1-type activity being higher than NAT2-type acetylation activity (Table 1). These data strongly suggest that functional NAT enzymes are present in lens epithelial cells and that NAT1 activity may be predominant. Therefore, these results suggest that NAT-dependent biotransformation pathway occurs in HLE cells.

Peroxynitrite and SIN1-Generated Peroxynitrite Irreversibly Inactivate NAT1 in Human Lens Epithelial Cells. Oxidative stress in lens cells is known to make a key contribution to cataract formation (Spector, 1991; Halliwell and Gutteridge, 1999). In addition, increases in the levels of oxidants in HLE cells are known to cause the oxidation-dependent inhibition of several key lens enzymes (Reddy et al., 2001; Hosler et al., 2003; Rogers et al., 2004; Thiagarajan et al., 2004). We thus used HLEB3 cells to analyze the effect on endogenous NAT1 and NAT2 enzymes of peroxynitrite, a cellular oxidant produced endogenously in various tissues of the eye and involved in cataract formation (Thiagarajan et al., 2004). HLEB3 cells have been widely used as a model for in vivo studies of HLE cell physiology, eye-related toxicology, and cataract (Andley et al., 1994; Hosler et al., 2003).

We assessed the sensitivity of endogenous NAT1 activity to peroxynitrite-dependent inactivation by exposing HLEB3 cell monolayers to increasing concentrations of peroxynitrite (100–400 μM). We also mimicked physiological peroxynitrite generation by exposing these cells to SIN1, a compound that releases peroxynitrite (Singh et al., 1999; Daiber et al., 2000). SIN1 has been shown to attack many biological targets in the same manner as authentic peroxynitrite and has thus been widely used as a source of peroxynitrite in studies on cultured cells (Singh et al., 1999). The concentrations of peroxynitrite used in our study are well within the physiological/pathophysiological range. Thus, a bolus of 250 μM peroxynitrite is equivalent to the constant exposure to a concentration of 1 mM peroxynitrite for only 7 min (Stachowiak et al., 1998). The physiological/pathophysiological concentrations of peroxynitrite in vivo has been estimated to range from ~50 to ~500 μM (Stachowiak et al., 1998; Mihm and Bauer, 2002). The exposure of HLEB3 cells to both exogenous peroxynitrite (Fig. 2, top, left) and SIN1-released peroxynitrite (top, right) caused significant concentration-dependent inactivation of the endogenous NAT1 enzyme. After 10-min exposure to a final concentration of 100 μM peroxynitrite, ~50% of cellular NAT1 was inactivated (top, left). Cellular NAT1 was completely inactivated by exposure for 10 min to 400 μM peroxynitrite. Exposure for 30 min to SIN1, which releases peroxynitrite continuously, led to the weaker, but significant inactivation (50% with 1 mM SIN1) of cellular NAT1 (top, right). This weaker effect probably results from SIN1 generating smaller amounts of peroxynitrite than the parent peroxynitrite donor (at least 75% smaller; Dairou et al., 2004). Decomposed SIN1, which does not release peroxynitrite, had no effect on cellular NAT1 (data not shown). We investigated whether the peroxynitrite-dependent inactivation of NAT1 in cells was irreversible by incubating extracts from HLEB3 cells exposed to 400 μM peroxynitrite with DTT (10 mM final concentration) and measuring NAT1 activity. DTT, a potent reducing agent, did not significantly (<20%) reverse peroxynitrite-induced inactivation compared with 400 μM peroxynitrite without DTT (Fig. 2, top, right). These results suggest that human NAT1 is inactivated in HLE cells by physiologically/pathophysiologically relevant levels of peroxynitrite. Similar results were obtained when the NAT2 activity of HLE cells was studied except that total inactivation of NAT2 enzyme was obtained with 200 μM peroxynitrite and 2 mM SIN1 (data not shown).

Reversible Inhibition of NAT1 in Human Lens Epithelial Cells by Continuous Generation of H_2O_2. H_2O_2 is another major oxidant to which HLE cells may be exposed (Halliwell and Gutteridge, 1999; Dudek et al., 2001) and the H_2O_2-dependent modification of HLE cell proteins has been linked to cataract formation (Paron et al., 2004). In the lens, H_2O_2 concentrations may reach sublethal levels (up to 600 μM; von Kobbe et al., 2004). We thought it likely that NAT1 and NAT2 enzymes would be a potential target for reversible inactivation by H_2O_2 in HLE cells. To this end, we assessed the effect of H_2O_2 on endogenous NAT1 and NAT2 activity by exposing HLEB3 cells to H_2O_2 generated continuously by the steady conversion of β-D-glucose to D-gluconolactone and H_2O_2 catalyzed by GOX (Ravid et al., 2002). Indeed, in vivo

TABLE 1

NAT1- and NAT2-type acetylation activity in epithelial lens cells

NAT1- and NAT2-type acetylation activity in human epithelial lens cells (HLEB3 and FHL124 cells) and in rabbit epithelial lens cells was measured in normalized cell extracts by measuring the AcCoA-dependent acetylation of PAS (NAT1-type substrate) and SMZ (NAT2-type substrate). These results are representative of three independent experiments, in which activity was measured in quadruplicate. Results are presented as mean ± S.D. of acetylated PAS or SMZ.

	NAT1-Type Activity	NAT2-Type Activity
	nmol/mg/min	
HLEB3 cells	5.9 ± 0.4	0.8 ± 0.05
FHL124 cells	2.34 ± 0.9	0.6 ± 0.02
Rabbit LE cells	13.6 ± 1.5	2.4 ± 0.09

the rate of H_2O_2 generation, although different for various kinds of cells, is continuous. Hence, exposing cells to a continuous flow of H_2O_2, as opposed to bolus addition, represents a superior method of delivery that mimics physiological conditions (Mueller et al., 2001; Ravid et al., 2002). In the conditions used (Atmane et al., 2003), the constant rate of production of H_2O_2 was estimated at ~5 μM H_2O_2/min, which is physiologically/pathophysiologically relevant (Mueller et al., 2001; Ravid et al., 2002). Endogenous NAT1 was inhibited in a time-dependent manner by the continuous production of H_2O_2 via the glucose/glucose oxidase system (Fig. 3, left). After 10 min of exposure, endogenous NAT1 activity levels were close to 60% control levels. After 30 min of exposure to H_2O_2, residual NAT1 activity in HLEB3 cells was less than 30% control levels. No inactivation of endogenous NAT1 activity by H_2O_2 generated by the glucose/glucose oxidase system was observed in the presence of catalase (300 units/ml). We also investigated whether the inactivation of endogenous NAT1 by the continuous generation of H_2O_2 was reversible. Both high GSH (10 mM final) and DTT (10 mM final) concentrations reactivated significantly NAT1 in HLEB3 cells exposed for 30 min to continuously generated H_2O_2, with more than 95% of endogenous NAT1 activity reactivated by 10 mM DTT (Fig. 3B, top, right). Thus, NAT1 activity in HLE cells seems to be impaired by physiologically/pathophysiologically relevant levels of H_2O_2. Our results also suggest that the H_2O_2-inactivated NAT1 enzyme can be reactivated by high concentrations of reducing agents such as GSH and DTT. Similar results were obtained for the NAT2 isoform in HLE cells (data not shown).

Reversible Photooxidative Inhibition of NAT1 in Human Lens Epithelial Cells upon UVB Irradiation. UVB radiation is known to be involved in cataract formation, via the oxidation-dependent inactivation of various lens macromolecules (Rogers et al., 2004). Several lens enzymes are inactivated by UVB-dependent photooxidative processes (Reddy et al., 2001). We therefore also investigated the effect of physiologically relevant doses of UVB on the endogenous NAT1 enzyme of HLE cells. To this end, HLEB3 cells were irradiated with 100, 200, and 400 J/m^2 of UVB and then maintained in culture for 15 min at 37°C. As shown in Fig. 4, UVB irradiation of HLEB3 cells caused significant dose-dependent inactivation of the endogenous NAT1 enzyme (Fig. 4). Approximately 50% of cellular NAT1 was inactivated by 400 J/m^2 UVB irradiation. We investigated whether the UVB-dependent inactivation of NAT1 was caused by a reversible photooxidative process by treating extracts of HLEB3 cells with UVB irradiation (400 J/m^2) and incubating them with a high concentration of DTT (10 mM final). The complete recovery of NAT1 activity was observed (Fig. 4).

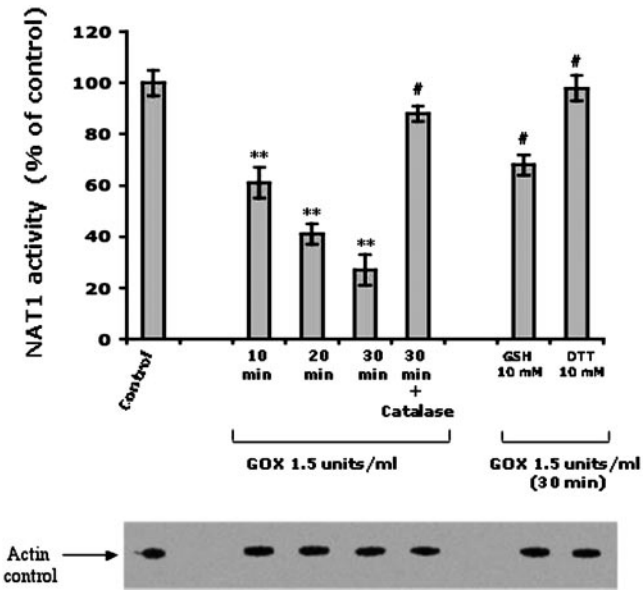

Fig. 3. Inhibition of endogenous NAT1 activity in HLEB3 human lens cells by the continuous generation of hydrogen peroxide. HLEB3 cells in Petri dishes were exposed to 1.5 U/ml GOX and 5 mM glucose in PBS or MEM at 37°C for the indicated periods of time. The rate of H_2O_2 production was constant in these experiments at ~5 μM H_2O_2/min (Atmane et al., 2003). A control reaction was carried out in the presence of 300 U/ml catalase (CAT). Cells were washed with PBS, scraped into lysis buffer, and a total cell extract was obtained after ultracentrifugation. NAT1 activity in normalized cell extracts was assessed using DMAB, as described previously (Sinclair et al., 1998) (top). Normalization of the cell extracts assayed was also checked by Western blotting using an anti-actin antibody (bottom). Extracts from HLEB3 cells exposed to PBS only were used as a control. We studied the reactivation by reducing agents of H_2O_2-inhibited NAT1, by incubating extracts from HLEB3 cells treated with GOX for 30 min with reducing agents (10 mM final concentration GSH or DTT) for 10 min at 4°C before measuring activity. Results are presented as percentage of NAT1 activity. Error bars indicate the S.D. values (**, $p < 0.01$ versus control; #, $p < 0.01$ versus NAT1 activity in extracts from 30 min GOX-treated HLEB3 cells).

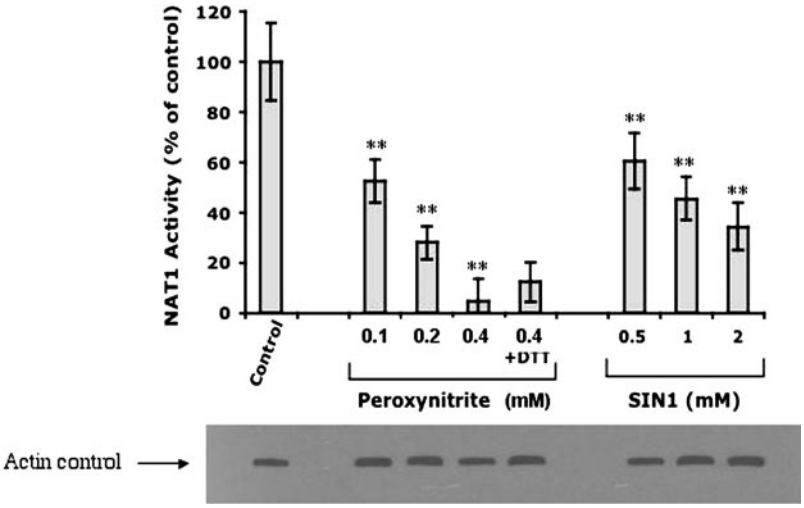

Fig. 2. Inactivation of endogenous NAT1 activity in HLEB3 human lens cells upon exposure to peroxynitrite or to peroxynitrite generated by SIN1. HLEB3 cells in Petri dishes were exposed to specified final concentrations of peroxynitrite or SIN1 in 10 ml of PBS for 10 min at 37°C. Cells were washed with PBS, scraped into lysis buffer, and a total cell extract was obtained after ultracentrifugation. NAT1 activity in normalized cell extracts was assessed using DMAB (top). Normalization of the cell extracts assayed was also checked by Western blotting with an anti-actin antibody (bottom). Extracts from HLEB3 cells exposed to PBS only were used as a control. We investigated the reactivation of peroxynitrite or SIN1-inhibited NAT1 by reducing agents. To this end, we incubated extracts of HLEB3 cells treated with 400 μM peroxynitrite or 2 mM SIN1 with reducing agent (10 mM DTT) for 10 min at 4°C before measuring activity. The results presented are the means of three independent experiments in which each treatment was performed in triplicate. Activity was measured in quadruplicate. Results are presented as percent NAT1 activity. Error bars indicate S.D. values (**, $p < 0.01$ versus control).

Thus, the exposure of HLE cells to subsolar levels of UVB leads to the reversible inactivation of endogenous NAT1 through a photooxidative process. Similar results were obtained for NAT2 in HLE cells (data not shown).

Discussion

Age-dependent cataract is a major ocular disease causing blindness. It is now known that increases in the levels of oxidants in lens cells via various oxidative processes, such as UV irradiation, exposure to toxic chemicals, inflammation, or impairment of antioxidant defense systems, lead to lens opacification and cataract formation through protein modification (Truscott, 2003). Age-dependent cataract formation is also generally thought to result from an interaction between genetic and environmental factors. Indeed, chemical compounds present in tobacco smoke and therapeutic drugs may display ocular toxicity and contribute to cataract development (Solberg et al., 1998; To and Towsend, 2000). The arylamine NATs detoxification pathway was recently suggested to play a protective role in lens cells (Meyer et al., 2003). Indeed, NAT2 slow acetylators have been shown to be at higher risk of developing age-dependent cataract than fast acetylators, suggesting that exogenous chemical factors detoxified by NATs may act as etiological agents for cataract formation (Meyer et al., 2003). It is noteworthy that tobacco smoke, which is known to be involved in cataract formation, contains aromatic amines, such as 4-aminobiphenyl, that are detoxified by NATs (Hein et al., 2000). In addition, therapeutic drugs detoxified by NATs, such as sulfamides and isoniazid, have been implicated in cataract formation (Van Den Brule et al., 1998) and ocular toxicity (To and Towsend, 2000). Although the hepatic xenobiotic metabolism may have an impact on lens through transport of parent chemicals and/or their metabolites, expression of XMEs in lens cells may be important for the lens homeostasis through in situ detoxification of toxic compounds. We therefore investigated whether NAT enzymes were present and active in HLE cells, which are cells essential for maintenance of the metabolic homeostasis and transparency of the entire lens (Spector, 1991). We demonstrate here, by RT-PCR, Western blotting, and enzyme assays that the functional NAT1 enzyme is present in HLE cells. RT-PCR revealed that the NAT2 mRNA was also present in HLE cells, but the corresponding protein was not detected by immunoblotting. We detected a lower but reproducible NAT2 activity compared with NAT1. Such a difference between the expression profiles of NAT1 and NAT2 has been reported for other cell types (Kawakubo et al., 2000). The mRNAs encoding NAT1 and NAT2 have been found in liver, gastrointestinal tract tissues, ureter, bladder, lung (Windmill et al., 2000), and mammary gland (Sadrieh et al., 1996). However, NAT2 protein has been detected only in liver, colon, and periodontal tissues (Sim et al., 2000; Meisel et al., 2002), whereas NAT1 protein is readily detected in most tissues (Sim et al., 2000; Rodrigues-Lima et al., 2003). The lack of detection of NAT2 protein in HLE cells in this study may therefore be caused by the production of only small amounts of protein or to the anti-NAT2 antibody having a lower titer than the anti-NAT1 antibody (Stanley et al., 1996). However, given that NATs are polymorphic enzymes and that differences in immunoreactive recombinant protein variants have been reported in yeast expression systems, we cannot rule out that the lack of detection of the NAT2 protein may be the result of NAT2 genotypes leading to low NAT2 protein expression (Fretland et al., 2001). We also detected the counterparts of the human NAT1 and NAT2 enzymes in rabbit epithelial lens cells, using NAT1 and NAT2 substrates (Table 1). As in HLE cells, NAT1-type activity was higher than NAT2 in rabbit lens epithelial cells (Table 1). These results are consistent with previous data obtained with rabbit eye tissues (Campbell et al., 1991). In addition, NAT1- and NAT2-type activities have also been previously found in bovine lenses, with NAT1-type activity being higher than NAT2 (Gaudet et al., 1995). Overall, our results suggest that functional NAT enzymes are present in HLE cells. These data support the hypothesis that NAT-dependent detoxification occurs in human lens cells. Other XMEs, including glutathione *S*-transferases (Raghavachari et al., 1999) and NADPH:quinone oxidoreductase (Tumminia et al., 1993), have also been found in lens cells, suggesting that the detoxification processes catalyzed by these enzymes take place in these cells. Therefore, as suggested by Meyer et al. (2003), presence of NATs in HLE cells may play a protective role important for lens homeostasis by detoxifying xenobiotics, exposure to which over time could be toxic for the lens.

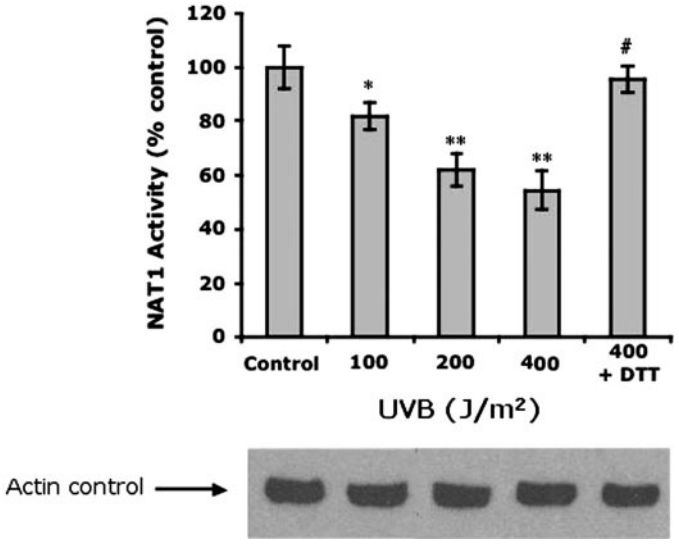

Fig. 4. Inhibition of endogenous NAT1 activity upon UVB irradiation of HLEB3 human lens epithelial cells. HLEB3 cells in Petri dishes were exposed to various doses of UVB irradiation in 10 ml of PBS or MEM. After 15 min at 37°C, cells were scraped into lysis buffer, and a total cell extract was obtained after ultracentrifugation. NAT1 activity in normalized cell extracts was assessed using DMAB as described previously (Sinclair et al., 1998) (top). Normalization of the cell extracts assayed was also checked by Western blotting with an anti-actin antibody (bottom). Extracts from HLEB3 cells exposed to PBS only were used as a control. We studied the reactivation of UVB-inhibited NAT1 by reducing agents, by incubating extracts from 400 J/m^2 UVB-treated HLEB3 cells with reducing agent (10 mM DTT) for 10 min at 4°C before measuring activity. The results presented are the means of three independent experiments in which each treatment was performed in triplicate. Activity was measured in quadruplicate. Results are presented as percent NAT1 activity. Error bars indicate the S.D. values (*, $p < 0.05$ versus control; **, $p < 0.01$ versus control; #, $p < 0.01$ versus NAT1 activity in extracts from 400 J/m^2 UVB-treated HLEB3 cells).

The generation of cellular oxidants and the subsequent oxidative modification of lenticular proteins play a fundamental role in age-dependent cataract development (Spector, 1991; Halliwell and Gutteridge, 1999; Davies and Truscott,

2001; Paron et al., 2004). Low levels of oxidants are continually produced in cells as a result of normal metabolic activity. However, high levels of oxidants may be produced in HLE cells because of UV-dependent photooxidative processes, exposure to toxic chemicals, or failure of antioxidant defense systems (Spector, 1991; Halliwell and Gutteridge, 1999; Dudek et al., 2001; Paron et al., 2004). Therefore, although lens cells produce large amounts of antioxidant molecules, such as superoxide dismutase and GSH, acute or chronic oxidative stress may nonetheless occur, leading to the oxidative damage of lens macromolecules over time (Halliwell and Gutteridge, 1999). Because oxidative damage to protective enzymes such as proteasome and catalase in HLE cells may be involved in cataract formation (Reddy et al., 2001; Hosler et al., 2003), we also investigated whether the activity of NAT enzymes in lens cells was impaired by oxidative and photooxidative stresses. Indeed, NAT enzymes can be inactivated by the oxidative modification of their catalytic cysteine residue (Rodrigues-Lima and Dupret, 2004). We clearly show here that endogenous NAT1 in HLE cells is inactivated by exposure of these cells to physiological/pathophysiological amounts of peroxynitrite or H_2O_2. These two major cellular oxidants are known to cause acute or chronic protein oxidation and enzymatic inactivation in HLE cells, thereby contributing to cataract formation over time (Paron et al., 2004; Thiagarajan et al., 2004). The inactivation of endogenous NAT1 in HLE cells by peroxynitrite was irreversible, whereas H_2O_2 caused an inactivation reversible on addition of high levels of reducing agents, such as GSH. These results are consistent with previous data obtained using recombinant NAT1 (Atmane et al., 2003; Dairou et al., 2003, 2004), suggesting that the inactivation of NAT1 in HLE cells is caused by the oxidative modification of its catalytic cysteine residue to sulfinic/sulfonic forms (irreversible inactivation) or to sulfenic/disulfide forms (reversible inactivation) (Rodrigues-Lima and Dupret, 2004). Other key enzymes, such as the proteasome and glyceraldehyde-3-phosphate dehydrogenase, are inactivated in HLE cells by the oxidative modification of important residues upon exposure of the cells to oxidants or UVB (Hosler et al., 2003; Paron et al., 2004). Photooxidative damage of HLE cell proteins as a result of UVB irradiation is also known to be involved in cataract formation (Halliwell and Gutteridge, 1999; Davies and Truscott, 2001; Reddy et al., 2001). We show here that endogenous NAT1 is inactivated if HLE cells are exposed to physiologically meaningful doses of UVB. This inactivation is reversed by high concentrations of reducing agents such as GSH, suggesting that the inactivation of NAT1 in HLE cells by UVB is probably caused by oxidative modification of the catalytic cysteine residue to a sulfenic or disulfide form (Rodrigues-Lima and Dupret, 2004). Likewise, protein tyrosine phosphatases, which also possess a catalytic cysteine residue, have been shown to be reversibly inactivated in cells upon UVB irradiation, this inactivation being a key event in the UV-induced signal transduction (Grob et al., 1999). In addition, in lens cells, aldose reductase, glucose-6-phosphate dehydrogenase, and catalase have been shown to be reversibly inactivated by UVB irradiation (Reddy et al., 2001; Rogers et al., 2004). Moreover, it is known that large amounts of oxidants are produced in cells as by-products of UV exposure (Halliwell and Gutteridge, 1999; Paron et al., 2004; Rogers et al., 2004). Thus, UVB radiation seems to cause oxidative damage to NAT1 in HLE cells, leading to the inactivation of this enzyme. We also investigated whether endogenous NAT2 activity in HLE cells was impaired by exposure to peroxynitrite, H_2O_2 or UVB. We obtained results similar to those for NAT1 (data not shown). These data are consistent with the high level of similarity between the two enzymes (81% amino acid sequence identity) and with the presence of the same catalytic cysteine residue in both enzymes (Dupret and Grant, 1992). Our results clearly show that the highly protective reducing environment of HLE cells (Halliwell and Gutteridge, 1999) may nonetheless be insufficient to protect endogenous NAT enzymes from oxidative and photooxidative stress-dependent inactivation and provide further evidence that NAT enzymes are highly sensitive to oxidative stress in cells (Rodrigues-Lima and Dupret, 2004). Finally, our results support the hypothesis that the NAT biotransformation pathway may play a protective role in the maintenance of lens homeostasis via the detoxification of xenobiotics to which exposure could be deleterious for the lens over time (Meyer et al., 2003). Consequently impairment of the activity of these key XME in lens cells by oxidative and photooxidative processes may contribute to cataract formation.

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