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Induction of NEIL1 and NEIL2 DNA glycosylases in aniline-induced splenic toxicity

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Abstract

The mechanisms by which aniline exposure elicits splenotoxic response, especially the tumorigenic response, are not well-understood. Earlier, we have shown that aniline-induced oxidative stress is associated with increased oxidative DNA damage in rat spleen. The base excision repair (BER) pathway is the major mechanism for the repair of oxidative DNA base lesions, and we have shown an up-regulation of 8-oxoguanine glycosylase 1 (OGG1), a specific DNA glycosylase involved in the removal of 8-hydroxy-2'-deoxyguanosine (8-OHdG) adducts, following aniline exposure. Nei-like DNA glycosylases (NEIL1/2) belong to a family of BER proteins that are distinct from other DNA glycosylases, including OGG1. However, contribution of NEIL1/2 in the repair of aniline-induced oxidative DNA damage in the spleen is not known. This study was, therefore, focused on evaluating if NEILs also contribute to the repair of oxidative DNA lesions in the spleen following aniline exposure. To achieve that, male SD rats were subchronically exposed to aniline (0.5 mmol/kg/day via drinking water for 30 days), while controls received drinking water only. The BER activity of NEIL1/2 was assayed using a bubble structure substrate containing 5-OHU (preferred substrates for NEIL1 and NEIL2) and by quantitating the cleavage products. Aniline treatment led to a 1.25-fold increase in the NEIL1/2-associated BER activity in the nuclear extracts of spleen compared to the controls. Real-time PCR analysis for NEIL1 and NEIL2 mRNA expression in the spleen revealed 2.7- and 3.9-fold increases, respectively, in aniline-treated rats compared to controls. Likewise, Western blot analysis showed that protein expression of NEIL1 and NEIL2 in the nuclear extract of spleens from aniline-treated rats was 2.0- and 3.8-fold higher than controls, respectively. Aniline treatment also led to stronger immunoreactivity for NEIL1 and NEIL2 in the spleens, confined to the red pulp areas. These studies, thus, show that aniline-induced oxidative stress is associated with an induction of NEIL1/2. The increased NEILs-mediated BER activity is another indication of aniline-induced oxidative damage in the spleen and could constitute another important mechanism of removal of oxidative DNA lesions, especially in transcribed DNA following aniline insult.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

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Keywords

Spleen; Oxidative DNA damage; NEIL1/2; DNA repair; Aniline; Base excision repair

Introduction

Aniline, a toxic aromatic amine, is an extensively used industrial chemical with an annual production of over 1 billion pounds in the United States (Di Girolamo et al., 2009). Aniline exposure, besides inducing methemoglobinemia, hemolysis and hemolytic anemia (Jenkins et al., 1972; Harrison and Jollows, 1987; Mier, 1988;Pauluhn, 2004), is also associated with damage to the spleen, which is characterized by splenomegaly, increased erythropoietic activity, hyperpigmentation, hyperplasia, fibrosis, and a variety of primary sarcomas of the spleen after chronic exposure in rats (Goodman et al., 1984; Weinberger et al., 1985; Bus and Popp, 1987; Khan et al., 1993, 1997, 1999, 2006; Pauluhn, 2004; Ma et al., 2008). However, the molecular mechanisms by which aniline exerts its toxic effects in the spleen, especially the formation of various types of sarcomas and/or tumors need to be examined in view of occupational and environmental human exposure to aniline and related chemicals (Albrecht and Neumann, 1985; Bus and Popp, 1987; Di Girolamo et al., 2009). Reactive oxygen species (ROS) are believed to play an important role in the pathogenesis of several diseases including cancer, rheumatoid arthritis, cardiovascular disease, and aging (Ames et al., 1993; Gotz et al., 1994). ROS are formed endogenously as a byproduct of respiration and oxidative metabolism, and exogenously by a variety of environmental agents. Earlier studies from our laboratory have shown that aniline exposure is associated with iron overload and oxidative stress in the spleen (Khan et al., 1997, 1999, 2003a, 2003b). More importantly, our studies have shown increased oxidative DNA damage in the spleen of rats following aniline exposure (Wu et al., 2005; Ma et al., 2008), which could potentially lead to mutagenic and/or carcinogenic responses in the spleen.

To protect cells from oxidative DNA damage and mutagenesis, organisms possess multiple glycosylases which recognize the damaged bases and initiate the base excision repair (BER) pathway (Liu et al., 2010). The DNA BER pathway is the major pathway by which oxidative DNA lesions are removed from the genome, thus, representing a critical step in the maintenance of genome stability. Accordingly, this pathway is critical for preventing diseases resulting from oxidative DNA damage.

In mammalian cells, there are at least five different DNA glycosylases with overlapping substrate specificities that remove oxidative DNA base lesions. These include the Nei-like DNA glycosylases (NEIL1/2/3), 8-oxoguanine glycosylase 1 (OGG1) and endonuclease three homologue 1 (NTH1) (Rolseth et al., 2008; Mori et al., 2009). We have previously shown that increased oxidative DNA damage was associated with an up-regulation of OGG1, a specific DNA glycosylase involved in the removal of 8-hydroxy-2'-deoxyguanosine (8-OHdG) adducts, in the spleen of rats exposed to aniline (Ma et al., 2008). NEILs are distinct from OGG1 in structural features and reaction mechanisms while they act on many of the same substrates (Dou et al., 2003; Englander and Ma, 2006). NEIL1/2, unlike OGG1 and NTH1, are able to excise oxidized base lesions from single-stranded DNA regions which suggests their preferential involvement in the repair of DNA damage during replication and/or transcription (Dou et al., 2008). Hence NEIL1/2 may play a unique role in maintaining the functional integrity of mammalian genomes. Even though aniline exposure leads to oxidative DNA damage, and up-regulation of OGG1, essentially nothing is known about the status and contribution of NEILs in the DNA repair process in the spleen following aniline insult. In the current study, we test the hypothesis that aniline

exposure also provokes NEIL1/2-mediated BER in rat spleen, in addition to OGG1-mediated BER.

Materials and methods

Animal and treatment

Male Sprague-Dawley rats (~200 g), obtained from Harlan (Indianapolis, IN), were housed in wire-bottom cages over absorbent paper with free access to tap water and Purina rat chow. The animals were acclimatized in a controlled-environment animal room (temperature, 22 °C; relative humidity, 50%; photoperiod, 12-h light/dark cycle) for 7 days prior to treatment. The experiments were performed in accordance with the guidelines of the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee of University of Texas Medical Branch. The animals were divided into two groups of six rats each. One group of animals was given 0.5 mmol/kg/day (~64.7 mg/kg/day) of aniline hydrochloride (~97%; Sigma-Aldrich, Milwaukee, WI) via drinking water (pH of the solution adjusted to ~6.8) (Khan et al., 1999; Ma et al., 2008, Wang et al., 2008), whereas the other group received drinking water only and served as controls. Choice of dose and duration of exposure was based on earlier studies (Khan et al., 1999; Wang et al., 2005; Ma et al., 2008). After 30 days, the rats were euthanized under nembutal (sodium pentobarbital) anesthesia and the spleens were removed immediately, blotted, weighed and stored at -80 °C until further analysis. A portion of spleen was snap frozen in liquid nitrogen and stored at -80 °C for RNA isolation. Also, portions of the spleen from control and aniline-treated rats were fixed in 10% neutral buffered formalin for immunohistological processing.

Preparation of nuclear extracts (NEs) for NEIL activity and Western blotting

The nuclear protein extracts (NEs) were prepared according to the method of Ma et al. (2008), with minor modifications. Spleen tissues (from control and aniline-treated rats) were cut into smaller pieces, homogenized briefly with a loose glass pestle in cold hypotonic buffer [10mM HEPES-KOH, 10mM KCl, 100 μM EDTA, 100 μM EGTA, 1 mM DTT, 0.5 mM PMSF, 2μg/ml pepstatin, and a complete protease inhibitor cocktail (Roche, Germany)], and incubated on ice for 20 min. Tissues were then homogenized with a tight pestle and centrifuged at 800g for 4 min to obtain nuclear pellets. Pellets were gently washed two times with homogenizing buffer. Nuclear proteins were extracted in a high salt buffer (20mM HEPES -KOH, 405mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 2 μg/ml pepstatin, and protease inhibitor cocktail) by incubating for 45 min on ice with reverse mixing at intervals of 10min. The NEs were cleared by centrifugation (16,000 g, 10 min) and adjusted to 15 % with glycerol and stored at -80°C until further analysis.

Oligonucleotide 5' end-labeling for NEIL1/2-mediated BER assay

A 51-mer oligonucleotide containing 5-OHU at position 26 from the 5'-end (sequence shown below) were obtained from Sigma (St. Louis, MO). Oligonucleotides (5.5 pmol) were end-labeled using 5 μCi of [γ -³²P]ATP (3000Ci/mmol, Perkin -Elmer Life & Analytical Sciences, Boston, MA) and T4 polynucleotide kinase (New England BioLabs, Ipswich, MA) and then passed through a G-25 spin column (GE Healthcare, Piscataway, NJ) for purifying the radio labeled oligonucleotides, and annealed with 1.5–2 fold complementary oligonucleotide by gradual cooling to room temperature. 5-OHU(U): bubble:
Oligonucleotide substrate sequence *5'-GCT TAG CTT GGA ATC GTA TCA TGT AUA CTC GTG TGC CGT GTA GAC GAT GCC-3' 3' CGA ATC GAA CCT TAG CAT AG GCACCCGACAA AC ACG GCA CAT CTG CTA CCG-5'

NEILs cleavage assay

Glycosylase activities were measured *in vitro* in the NEs with synthetic end-labeled double-stranded oligonucleotide substrates containing 5-OHU(U): bubble (targeted by NEIL1 and NEIL2). Glycosylase assays were done as described earlier (Ma et al., 2008) with slight modifications. Briefly, the reactions were done in 20 μ l with 40 μ g NEs, 1 nM end-labeled substrate, and reaction buffer (10mM HEPES -KOH, pH 7.6, 50mM KCl, 1.0 mM DTT, 1.5% glycerol, 2.25 mM EDTA), and the final NaCl concentration was adjusted to 85 mM. Incubation was done at 37°C for indicated times and was terminated with 5X alkaline loading buffer (0.5 M NaOH, 97% formamide, 10 mM EDTA, pH 8, 0.025% bromophenol blue, 0.025% xylene cyanol) and heated at 90°C for 4 min. Positive control reactions were assembled using purified NEIL2 enzyme (Hazra et al., 2002a). Reaction mixtures were resolved on 15% polyacrylamide-7 M urea gels in Tris-borate buffer (89 mM Tris-HCl, 89 mM H₃BO₃, 2 mM EDTA, pH ~ 8.3) at 16 mA for 150 min, and products were visualized by autoradiography and quantified on Phosphorimager (GE Healthcare). Phosphorimager values, representing percent of substrate cleavage within the linear range of each reaction, were converted into cleavage-product amounts. Values from 6 individual rats and 3 cleavage assays per extract were averaged and plotted as means \pm SD.

RNA isolation, cDNA synthesis and real-time PCR for NEIL1 and NEIL2

Total RNA isolation from spleen tissues, and cDNA synthesis and the real-time PCR were performed as described in our earlier study (Ma et al., 2008) with minor modifications.

RNA isolation—Total RNA was isolated from spleen tissues using RiboPure kit (Ambion, Austin, TX) as per the manufacturer's instructions. To eliminate contaminating genomic DNA, RNA preparation was treated with RNase free DNase I (DNA-free kit, Ambion, Austin, TX). The total RNA concentration was determined by measuring the absorbance at 260 nm. RNA integrity was verified electrophoretically by ethidium bromide staining and by measuring A260/A280 ratio.

Real-time PCR—The real-time PCR was performed essentially as described earlier (Wang et al., 2005; Ma et al., 2008). Briefly, first-strand cDNA was prepared from isolated RNA by using SuperScript III First-Strand Synthesis Kit (Invitrogen, Carlsbad, CA) as described earlier (Wang et al., 2005). Quantitative real-time PCR employing a two-step cycling protocol (denaturation and annealing/extension) was carried out using Mastercycler Realplex as per manufacturers instructions (Eppendorf North America, Westbury, NY). The sequences of the forward and the reverse primers of NEIL1 and NEIL2 for real-time PCR were 5'-ACACCCTGCTGATTTGCTA-3' and 5'-GAGGCCAGCTTGGTCTACAG-3' for NEIL1, and 5'-GGAATGTGGCAGAAAGAAGC-3' and 5'-GAGATCGTAAGGGCCATCAG-3' for NEIL2, respectively. For each cDNA sample, parallel reactions were performed in triplicate for the detection of and NEIL1, NEIL2 and 18 S. The reaction samples in a final volume of 25 μ l contained 2 μ l of cDNA templates, 2 μ l primer pair, 12.5 μ l iQ SYBR Green Supermix and 8.5 μ l water. Amplification conditions were identical for all reactions: 95°C for 2 min for template denaturation and hot start prior to PCR cycling. A typical cycling protocol consisted of three stages: 15 s at 95°C for denaturation, 30 s at 63 °C for annealing, 30s at 72 °C for extension, and an additional 6s hold for fluorescent signal acquisition. To avoid the non-specific signal from primer-dimers, the fluorescence signal was detected 2 °C below the melting temperature (T_m) of individual amplicon and above the T_m of the primer-dimers (Rajeevan et al., 2001 and Simpson et al., 2000). A total of 40 cycles were performed for the studies. After the final cycle of the PCR, a melt curve analysis (Ririe et al., 1997 and Wei et al., 2003) was performed for each sample. The reactions were cooled to 60 °C and then heated up to 95 °C at the ramp rate of 0.2 °C/s to denature double-stranded PCR products. The fluorescent

signal recorded during DNA melting was plotted against temperature to generate the melt curve for each reaction. The T_m of individual amplicon was identified from the melt curve analysis using the Mastercycler Realplex software (Eppendorf). The specificity and purity of an amplicon for a particular target gene was confirmed by electrophoresis on 2% agarose gel stained with ethidium bromide, which showed a single prominent band of expected size on gel.

Quantitation of PCR was done using the comparative C_T method as described in User Bulletin No. 2 of Applied Biosystems (Foster City, CA), and reported as fold difference relative to the calibrator cDNA (QuantumRNA Universal 18 S Standards, Ambion). The fold change in cDNA (target gene) of NEILs relative to the 18 S endogenous control was determined by: Fold change = $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T = (C_{T \text{ Aniline}} - C_{T \text{ 18S}}) - (C_{T \text{ Control}} - C_{T \text{ 18S}})$.

Western blotting for NEIL1 and NEIL2 in NEs

Protein extracts (60 μ g NEs) were denatured by heating at 90 °C for 5 min and subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, GenScript, Piscataway, NJ). The separated proteins were transferred onto polyvinylidene difluoride (PVDF) microporous membrane (Millipore Corporation, Billerica, MA) using a transfer buffer (25 mM Tris-HCl, 190mM glycine, pH 8.4, 10% m ethanol). The membrane was then blocked with 10% non-fat dry milk and incubated overnight at 4 °C with NEIL1 or NEIL2 antibodies (1:200 dilution; Santa Cruz, CA). The membrane was washed three times with PBS-Tween 20 buffer. After incubation with the secondary antibody [anti-goat IgG-horseradish peroxidase (HRP)], the membranes were washed and developed using a chemiluminescence detection kit (ECL, GE Healthcare). As a control, after stripping each membrane with stripping buffer (Boston BioProducts, Worcester, MA), the membrane was reprobbed with anti-actin antibody (Sigma) and developed as above.

Immunohistochemical localization of NEIL1 and NEIL2

Paraffin sections were cut and deparaffinized at 55°C for 1 h and treated with xylene and various concentrations of ethanol, and finally rehydrated with water (Ma et al., 2008). The slides were incubated with sodium citrate buffer at 95°C for 20 min for antigen retrieval and subsequently incubated with reagents for blocking the non-specific binding sites, which included quenching of endogenous peroxidase activity with 0.3% H_2O_2 in water for 15min, 10% normal serum (Sigma) for 30 min, and avidin and biotin block solutions (Vector Laboratories, Burlingame, CA) for 15min each. The sections were then incubated with primary antibody (NEIL1 antibody, 1:100, ProteinTech Group, Chicago, IL; NEIL2 antibody, 1:100, Santa Cruz) overnight at 4 °C. Immunoreactivity was detected by the ABC method (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA) with color development using the substrate 3,3'-diaminobenzidine (DAB). Mayer's Hematoxylin was then added as a counterstain for 1 min. The negative controls were immunostained as above, but with goat serum instead of primary antibodies. Histological evaluation of staining was done under an OLYMPUS BX51 Microscope (Leads Instruments, Inc., Irving, TX).

Statistical analysis

All data are expressed as means \pm SD of six animals in each group. Comparison between the groups was made by p value determination using Student's two-tailed t -test (GraphPad InStat 3 software, La Jolla, CA). A p value of <0.05 was considered to be statistically significant.

Results

Effect of aniline exposure on DNA base repair activity (BER) of the spleen

The capacity for incision of 5-OHU with end-labeled oligonucleotide bubble substrate (targeted by NEIL1 and NEIL2) was evaluated in the NEs from control and aniline-treated rats. The cleavage activities were calculated from the cleavage yields generated in the linear range of each reaction. NEIL1/2-mediated BER activities of NEs were determined by quantifying radioactivity generated over time in cleavage products of end-labeled oligonucleotides (Fig. 1A). The pattern of excision activities in NEs at 12, 24, 48, and 96 min was 0.70, 0.58, 0.45, and 0.30 fmol/ μ g protein/h, respectively, for control animals, whereas for aniline-treated animals it was 0.83, 0.76, 0.56, and 0.36 fmol/ μ g protein/h, respectively. The percent cleavage over time is shown in Fig. 1B. Our data, thus, show an increase of 1.25-fold in NEILs BER activities in the splenic NEs of aniline-treated rats, suggesting increased capacity to excise 5-OHU incorporated into bubble structured substrate, which was shown to be preferentially targeted by NEILs (Dou et al., 2003, Englander and Ma, 2006, Yndestad et al., 2009).

mRNA expression of NEILs

To determine the impact of aniline exposure on the expression of NEILs in the spleen, NEIL1 and NEIL2 mRNA levels were analyzed by real-time PCR. As shown in the Fig. 2, aniline exposure resulted in a 2.7- and 3.9-fold increases in NEIL1 (A) and NEIL2 (B) mRNA expression compared to controls, respectively ($p < 0.05$).

NEILs protein expression in the NEs

To investigate whether gene expression levels are associated with increases in corresponding protein levels, NEILs protein expression in the spleens was also determined by Western blotting. As evident from Fig. 3, NEIL1 and NEIL2 protein expression in the NEs of aniline-treated rats were 2.0 and 3.8 fold higher, respectively, than the controls ($p < 0.05$).

Immunohistochemical assessment of NEIL 1 and NEIL2 in the spleen

Immunohistochemical studies for NEILs were also conducted to determine their expression and localization in the spleen of experimental animals. While control spleens showed sparse immunostaining for NEIL1 and NEIL2, significantly increased number of cells immunostained for both NEILs in the spleens of aniline-treated rats (Figs. 4A and 4B: NEIL1; 4C and 4D: NEIL2). The immunoreactivity for NEIL1/2 appeared predominantly in the red pulp areas of the spleen. NEIL2 showed much stronger immunoactivity compared to NEIL1 following aniline insult with regard to both number of cells stained and overall staining intensity.

Discussion

Spleen performs several important functional roles, including filtration of blood, participation in immune responses, phagocytosis and removal of infectious organisms and damaged RBCs. Therefore, one serious consequence of any damage/injury to the spleen would lead to its reduced ability to perform its important functions. Despite the well-documented splenotoxic effects and tumorigenic responses resulting from exposure to aniline (Bus et al., 1987; Khan et al., 2006), in-depth studies providing mechanistic explanations for splenic toxicity are lacking. Our previous studies showed that aniline exposure was associated with increased oxidative DNA damage, manifested by activation of the OGG1-mediated BER pathway (Ma et al., 2008). In the current study, we provide evidence that exposure to aniline is also associated with oxidative DNA damage repaired by NEIL1/2-mediated mechanisms. Our data show that aniline exposure is associated with

induction of NEIL1 and NEIL2 gene and protein expression and increases in their BER activities in the spleen.

Contribution of oxidative mechanisms, including oxidative DNA damage, is well established in the splenic toxicity of aniline (Khan et al., 1999, 2006; Wu et al., 2005; Ma et al., 2008). Oxidative DNA damage is one of the most common threats to genomic stability, and is also associated with numerous pathological conditions, including cancer (Cooke et al., 2003; Gackowski et al., 2005; Ma et al., 2008). Therefore, repair of the modified bases by their removal from DNA is critical to prevent mutations. Aniline exposure in this study led to increased NEIL1/2-associated BER activity in the nuclear extract of spleens, clearly suggesting that NEILs have a role in the repair of oxidative DNA lesions in the spleen. We interpret the induction of NEILs in our study as an adaptive response to the increased oxidative stress/DNA damage reported earlier (Khan et al., 1999; Wu et al., 2005; Ma et al., 2008). In fact, our findings are supported by other studies which demonstrate activation of NEIL1 in response to reactive oxygen species (Das et al., 2005, 2007; Hatt et al., 2008).

While NEIL1 and NEIL2 have overlapping substrate specificities with both OGG1 and NTH1 (Yndestad et al., 2009), a unique property of NEILs is that they have high affinity in excising base lesions from single-stranded DNA or unrepaired sequences in bubble DNA (Dou et al., 2003). This distinct substrate specificity has given rise to their potential involvement in replication-associated and/or transcription-coupled repair (Englander and Ma, 2006; Hazra and Mitra, 2006; Mori et al., 2009). Furthermore, with respect to substrate specificity, while the two NEILs recognize 5-OHU (Hazra et al., 2002a, 2002b), only NEIL1 has an evident affinity to 8-oxoG in double stranded DNA (Morland et al., 2002) and prefers foramidopyrimidines as substrates, whereas NEIL2 almost exclusively excises oxidation products of cytosine, namely 5-OHU and 5-hydroxycytosine. Our data, therefore, suggest that aniline exposure could induce a variety of oxidized DNA lesions including foramidopyrimidines as well as oxidation products of cytosine, in addition to 8-OHdG shown earlier (Wu et al., 2005; Ma et al., 2008). NEIL1 and NEIL2 also have unique specificity towards spiroiminodihydroantoin and guanidinohydroantoin lesions, which are more mutagenic, oxidized products of 8-oxoG (Hailer et al., 2005). Significance of NEILs is further substantiated by studies showing that embryonic mouse stem cells deficient in NEIL1 are sensitive to low levels of γ -radiation, indicating that NEIL1 is important for the cellular defense against DNA damage (Rosenquist et al., 2003). Also, NEIL1 and NEIL2 variants may affect their functions, leading to altered cell death and carcinogenesis. Study suggests that polymorphisms of the NEIL2 gene may be markers for risk progression of squamous cell carcinomas of the oral cavity and oropharynx (Zhai et al., 2008). Furthermore, mitochondrial DNA from NEIL1^{-/-} mice show increased level of steady-state DNA damage and deletions relative to wild-type controls (Vartanian et al., 2006).

The increases in NEIL BER activity was coupled with increases in NEIL1/2 gene and protein expression, suggesting an effect of aniline on transcriptional regulation of NEILs in the spleen. Our Western blot data indicate 2.0- and 3.8-fold increases in NEIL1 and NEIL2 expression, respectively, in the nuclear extracts of spleen from aniline-treated rats as compared to the controls. Interestingly, NEIL1 and NEIL2 mRNA expression in the spleen of aniline-treated rats also showed the same rate of fold-increase (2.6- and 3.8-fold increases, respectively). It is also clearly evident from our data that NEIL2 had much greater response in both mRNA and protein expression compared to NEIL1. These observations are also supported by our immunohistochemistry data which showed relatively stronger immunoreactivity for NEIL2 as compared to NEIL1 in the spleens, suggesting greater production of adducts that are substrates for NEIL2. Since NEIL2 shows cell cycle-independent expression (Hazra et al., 2002b), it might be specifically involved in the repair of transcribed rather than replicating DNA (Dou et al., 2008). These special features make

NEIL2 an attractive candidate for specialized repair of products of oxidative lesions in transcribed DNA in terminally differentiated, post-mitotic cells (Dou et al., 2003, 2008). Our data thus point to the possibility that NEILs may be involved in removal of oxidative lesions from transcribed DNA, contributing to the lesser accumulation of damage in promoter regions of transcribed genes.

To our knowledge, this is the first study to report induction of NEIL1/2-associated BER activity and NEIL1/2 gene up-regulation in the spleen following aniline exposure. Spleens from aniline-treated rats had greater NEIL1/2 activity, mRNA and protein levels, and strong NEIL immunoreactivity, especially in the red pulp areas. The increased BER activity of NEILs may represent an adaptive response against ROS-induced DNA damage resulting from aniline exposure, and could be an important mechanism for the removal of oxidative DNA lesions. Further evaluation of NEILs through dose- and time-dependent studies will shed more light on their contribution in the removal of oxidative DNA lesions in aniline - induced splenic toxicity.

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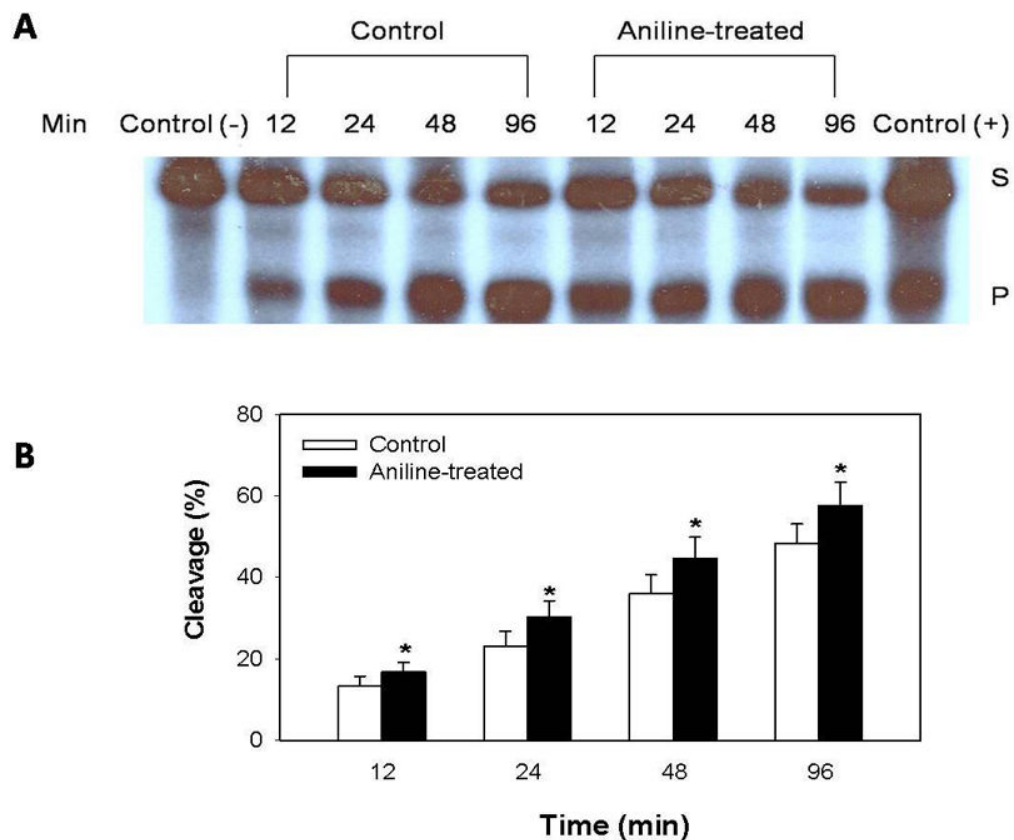


Fig. 1. BER activity in the splenic NEs of control and aniline-treated rats. The assay was conducted with end-labeled oligonucleotides (5-OHU:Bubble) which are targeted by NEILs (see methods for details). (A) Autoradiogram of incision products (P) generated over time by cleavage of end-labeled double-stranded oligonucleotides carrying the 5-OHU:Bubble adducts (S). Negative control (without NEs) and positive control (recombinant NEIL2) are included in the external lanes. Data represents 1 of the 5 samples assayed for BER activity in each group. (B) Values from Phosphorimager quantitation of products, generated with NEs from 5 rats per group and 3 cleavage assays per extract, were averaged and plotted as means \pm SD. * Indicate s that the cleavage is significantly different from controls for each respective time point ($p < 0.05$).

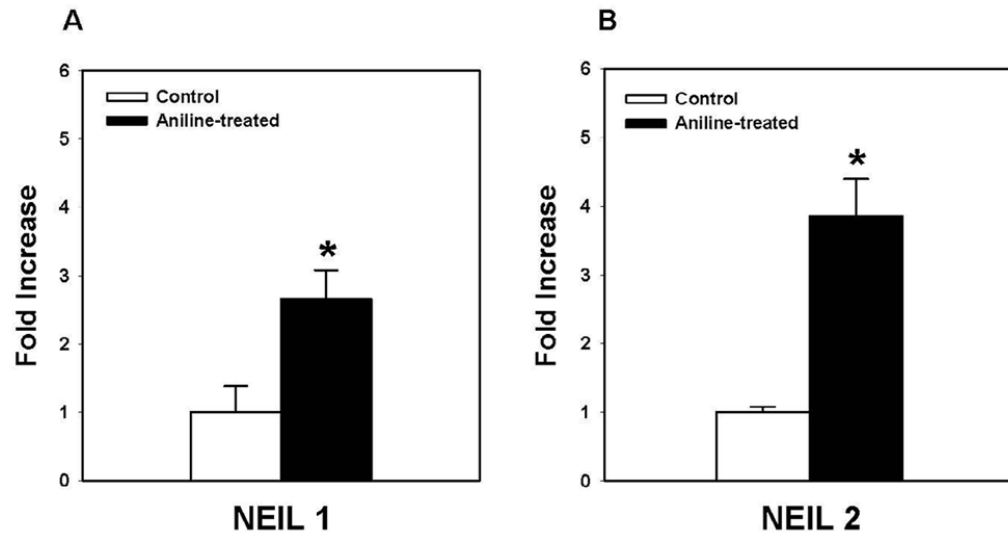


Fig. 2. Real-time PCR analysis of NEIL1 (A) and NEIL2 (B) gene expression in the spleens of control and aniline-treated rats. Total RNA was extracted from spleen tissues, real-time PCR was performed, and the fold change in mRNA expression was determined. Values are means \pm SD. * $p < 0.05$.

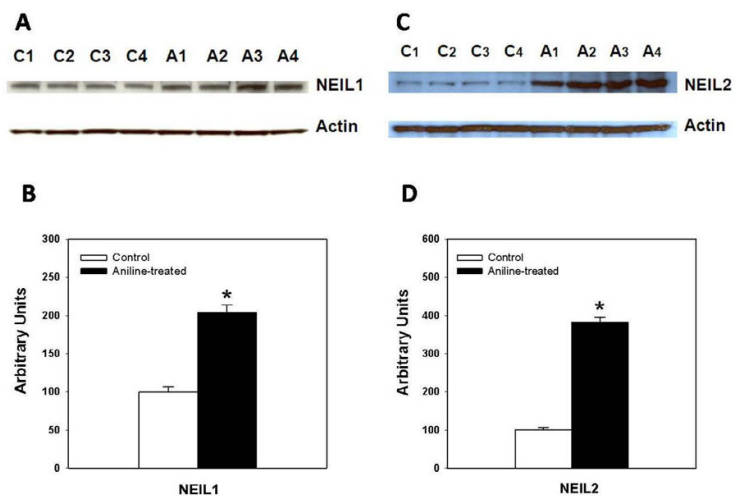


Fig. 3. Western blot detection of NEIL1(A) and NEIL2 (C) in the NEs from control and aniline-treated rats. Lanes C1–C4: controls; lanes A1–A4: aniline-treated. Densitometric analyses of NEIL1 and NEIL2 bands from control and aniline-treated rats (B, D). The densitometric analysis of the protein bands was done using Eagle Eye II software. Data from aniline-treated spleen samples were statistically compared to untreated controls, which were arbitrarily set at 100. Values are means \pm SD of four determinations. Protein expression of NEIL1 and NEIL2 in aniline-treated rats was 2.0- and 3.8-fold higher, respectively, in comparison to controls. * $p < 0.05$.

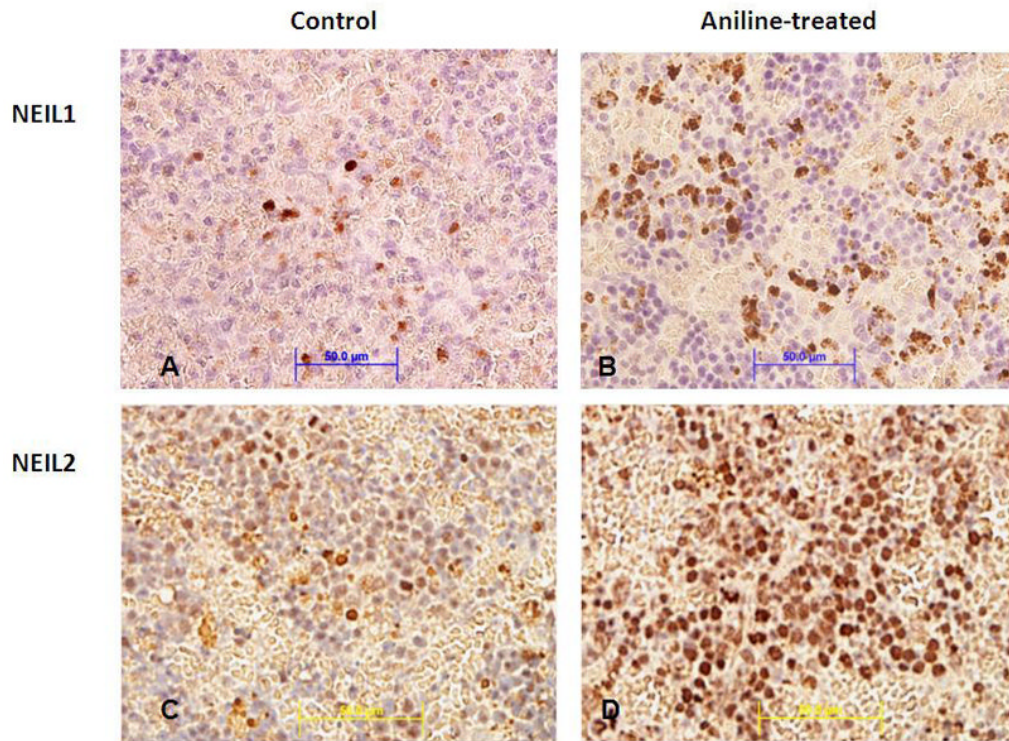


Fig. 4. Immunohistochemistry of NEILs in the spleens of control rats (A and C) vs. aniline-treated rats (B and D). Control spleen showed sparse immunoreactivity for NEIL1 and NEIL2, whereas spleen from aniline-treated rats showed increased immunoreactive cells and increased intensity of staining for NEIL1 and NEIL2, confined to the red pulp areas. Also, immunoreactivity for NEIL2 was relatively stronger than NEIL1, especially in aniline-treated spleen.