INDUCTION OF THE HUMAN OXIDIZED BASE-SPECIFIC DNA GLYCOSYLASE NEIL1 BY REACTIVE OXYGEN SPECIES

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Running Title: Oxidative stress induced NEIL1 activation

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NEIL1, a mammalian DNA glycosylase and ortholog of E. coli Nei/Fpg, is involved in the repair of oxidatively damaged bases in mammalian cells. Exposure of HCT116 human colon carcinoma cells to reactive oxygen species (ROS), generated by glucose oxidase (GO), enhanced the levels of NEIL1 mRNA and polypeptide by 2 – 4-fold by 6 h after GO treatment. A similar oxidative stress-induced increase in human NEIL1 (hNEIL1) promoter-dependent luciferase expression in HCT116 cells indicates that ROS activates NEIL1 transcription. The transcriptional start site of hNEIL1 was mapped, and the upstream promoter sequence was characterized via luciferase reporter assay. Two identical CRE/AP-1 binding sites were identified in the promoter that binds transcription factors c-Jun and CREB/ATF2. This binding was significantly enhanced in extracts of cells treated with GO. Further, simultaneous increase in the level of phosphorylated c-Jun suggests its involvement in upregulating the NEIL1 promoter. Oxidative stress-induced activation of NEIL1 appears to be involved in the feedback regulation of cellular repair activity needed to handle an increase in the level of oxidative base damage.

Introduction

ROS, generated endogenously and as a result of environmental insult, induce oxidative DNA damage which could affect the integrity of cellular genomes. Oxidative DNA damage, including a multitude of damaged bases, abasic (AP) sites and DNA strand breaks, is believed to be responsible for sporadic mutations leading to cancer and other pathological as well as age-related syndromes (1–4). Most of the oxidative damage in DNA is repaired via the base excision repair (BER) pathway, in which oxidized and altered bases are excised by DNA glycosylases in the first step of repair (5). BER is highly conserved among all organisms ranging from Escherichia coli to humans. In E. coli, three oxidatively damaged base-specific glycosylases -- endonuclease II (Nth), formamido (Fapy)-DNA glycosylase (Fpg) and endonuclease VIII (Nei) -- excise modified DNA bases with overlapping specificity. Based on structural similarity and reaction mechanism, these enzymes are classified in two families with Nth in one and Nei and Fpg in the other (6–8). In mammalian cells, only two DNA glycosylases, both belonging to the Nth family, were identified earlier for excision of oxidized bases. NTH1 removes mostly oxidized pyrimidines, e.g., thymine glycol (TG), while 8-oxoguanine DNA glycosylase (OGG1), functionally similar to Fpg but mechanistically analogous to Nth, is most active with 8-oxoguanine (8-oxoG) and Fapy-G substrates (9,10). Surprisingly, both NTH1- and OGG1-null mice are viable and show no major phenotype despite the known mutagenic and toxic effects of their substrate lesions (11,12). These results suggested the presence of additional DNA glycosylases in mammals that could serve as back-up enzymes for NTH1 and OGG1.

We and subsequently several other groups identified and characterized two additional human DNA glycosylases specific for oxidatively damaged bases, and named those NEIL1 and NEIL2 (13–17). Both of these enzymes belong to
the Fpg/Nei family; NEIL1 and NEIL2 share weak sequence similarity but have common characteristics, including overlapping substrate ranges (15-19). However, NEIL1 differs from NEIL2 due to its S-phase-specific activation while NEIL2 expression is independent of the cell cycle (13, 14). We have recently shown that both NEIL1 and NEIL2 possess an unusual ability to excise lesions from DNA bubble or single-stranded structures that could not be carried out by OGG1 or NTH1(20). This novel substrate structure preference of NEILs raises the possibility of their specialized in vivo functions for repair of oxidized bases in transient bubbles formed during replication and/or transcription.

Taken together, these results support the scenario that OGG1 and NTH1 are involved primarily in repair of oxidative damage formed in inactive sequences constituting the bulk of the genome. In contrast, NEIL1 and NEIL2 may be preferentially involved in repair of oxidized bases from the active sequences during replication or transcription, which should be more urgent than the repair of inactive sequences. In this report, we examined the possibility of modulation of NEIL1 activity and observed that human NEIL1 (hNEIL1) is activated by ROS. We then characterized the hNEIL1 promoter, and identified a pair of CRE/AP-1 sequences involved in the oxidative stress response.

Experimental Procedures

Cell Culture and Chemicals

The human colorectal carcinoma line HCT116 (with wild type p53), a gift of B. Vogelstein (John Hopkins University), was cultured in McCoy’s 5A modified medium supplemented with 10 % fetal bovine serum, 2 mM L-glutamine, penicillin (100 units/ml) and streptomycin (100 µg/ml; Gibco-BRL). At 70% confluence, the cells were treated with glucose oxidase (GO; Roche) at 100 ng/ml for 1 h, followed by washing with and incubation in fresh medium. This treatment did not affect cell viability, as judged by the rate of cell growth.

Measurement of Intracellular ROS Level

Changes in intracellular ROS level after glucose oxidase (GO) treatment were determined as described previously (21). Briefly, the cells were suspended in phosphate-buffered saline (PBS), and then treated with 5 µM 5-(and-6) carboxy-2′,7′-dichlorodihydro-fluorescein diacetate (H₂DCF-DA; Molecular Probes) for 15 min at 37°C. We determined in control experiments that cellular uptake of H₂DCF-DA reached a plateau at about 18 min when 0.6% of DCF, the oxidized form of H₂DCF, was released in the medium (data not shown). As a control, we also treated the cells with DCF, and observed a small increase in intracellular fluorescence (<5%) after glucose oxidase treatment (data not shown). The change in DCF fluorescence of treated vs. mock-treated cells was determined by flow cytometry (Becton Dickinson FACScan). The mean fluorescence for 12,000 cells, from three or more independent experiments, were analyzed, after correction for DCF extrusion in the medium, and expressed as ±SEM (Fig. 1).

Western Analysis

Cells were washed with ice-cold PBS, and lysed on ice for 30 min in a lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP40, 1 mM EDTA, 0.5 mM PMSF, and protease inhibitor cocktail; Roche) and then microfuged. Western analysis of the supernatant was carried out with antibodies to hNEIL1 (Alpha Diagnostics, San Antonio, TX), CREB, ATF2, c-Jun and phospho c-Jun (Santa Cruz Biotechnology), using enhanced chemiluminescence assay (ECL kit; Amersham Pharmacia); the signals were quantitated by densitometric scanning.

RT-PCR Analysis

RNA extraction was carried out with the RNeasy minikit (Qiagen) according to the manufacturer’s instructions. Total RNA (2-5 µg) was reverse transcribed using a Titan 1 step RT-PCR kit (Roche), followed by a limiting cycle amplification protocol using the following primer combinations: hNEIL1 forward, 5′-CGG CGG CTG CGT GGA GAA GTC-3′ and reverse, 5′-GTC CCA GCG GCC GAA CCG GCG-3′, designed to amplify a 300 bp region; GAPDH forward, 5′-GTG AAG GTC GGA GTC AAC-3′ and reverse, 5′-GGT GAA GAC GCC AGT GGA-3′, designed to amplify a 294 bp region (total number of cycles: 24 for hNEIL1 and 20 for GAPDH). The products of the semiquantitative RT-PCR were resolved in a 1.8% agarose gel, stained with ethidium bromide and photographed.

For quantitative, real time RT-PCR, one-step RT-PCR was performed with 100 ng cellular RNA for both the target gene and an endogenous
control in singleplex tubes using TaqMan one-step RT-PCR master mix reagent kit (P/N 4309169). The cycling parameters (for 40 cycles) in an ABI7000 thermal cycler were: reverse transcription at 48°C for 30 min, AmpliTaq activation 95°C for 10 min, denaturation 95°C for 15 sec and annealing/extension 60°C for 1 min. Duplicate Ct values were calculated using Microsoft Excel using a comparative Ct(ΔΔCt) method as described by the manufacturer (Applied Biosystems). The amount of target (2-ΔΔCt) was calculated after normalization to the 18s RNA as an endogenous reference, and relative to a calibrator (one of the experimental samples).

**Electrophoretic Gel Mobility Shift Analysis (EMSA)**

Nuclear extracts from HCT116 cells were prepared as described earlier (22). For EMSA, 5 µg nuclear extract was incubated with 2 pmol 5'-³²P-labeled duplex oligonucleotides and 1 µg poly [dI-dC]*[dI-dC] in a binding buffer containing 4% glycerol, 1 mM MgCl₂, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, and 10 mM Tris-HCl (pH 7.5) for 30 min at room temperature, followed by electrophoresis in 6% non-denaturing polyacrylamide gel in 0.5 X Tris/borate/EDTA (TBE) at 200 volt for 1 h. The gels were dried and subjected to Phosphorimager analysis. Competition of binding was performed in the presence of a 50- or 100-fold excess of the unlabeled oligonucleotide and incubating for 20 min at room temperature before adding the probe. Supershift assays were performed by preincubating the assay mixture with 4 µg of antibody against CREB/ATF2 or c-Jun for 1 h at 4°C before addition of ³²P-labeled oligo duplex probe.

**Genomic Cloning of the 5’ Upstream Region of hNEIL1**

An NdeI/XhoI fragment of the hNEIL1 cDNA was used to screen a BAC DNA library (Resgen) under stringent conditions. Restriction mapping and Southern blot analysis of BAC clones identified 18 kb HindIII, 13 kb EcoRI/HindIII, 12 kb BamHI/HindIII fragments which hybridized to the probe. The 13 kb EcoRI/HindIII fragment was further subcloned into pBluescript and sequenced. The sequences of the genomic clone were confirmed by matching with the published NEIL1 gene sequence (Genbank accession no. AAH10876). Analysis of candidate cis-elements to identify relevant transcription factors was performed using the programs TRANSFAC 4.0 (transfac.gbf.de/TRANSFAC) (23) and MatInspector V 2.2. Parameters for MatInspector were set for 1.0 core similarity (a 4 nucleotide highly conserved sequence) and 0.85 matrix similarity, employing the vertebrate matrix group.

** Primer Extension Analysis**

An antisense primer, P2 (5’-GGCTGAGGCAGGAATC-3’), complementary to 5'-untranslated region (UTR) of the hNEIL1 cDNA, near the beginning of exon 1, was 5’-³²P-labeled using [γ-³²P] ATP and T4 polynucleotide kinase, and annealed to 50 µg total RNA at 58°C for 20 min. The primer extension was performed with the annealed RNA DNA duplex, using 5 U AMV reverse transcriptase (Promega) at 42°C for 1 h in the supplied extension buffer and the products resolved in a 6% polyacrylamide sequencing gel. A control reaction was set up with a second antisense oligo P1, further downstream to the P2 sequence. Sequencing reactions, generated by Sanger’s dideoxy termination method, using primer P2 annealed to the hNEIL1 plasmid, were run in parallel to determine the exact length of the primer extension products.

**Plasmids, Transfection and Luciferase Assay**

A 2.1 kb fragment spanning the upstream sequence of hNEIL1, including part of the first exon, was subcloned from the 13 kb genomic clone at the XhoI/HindIII sites of the promoterless luciferase vector pGL2-basic (Promega), and the recombinant plasmid was named p (-2100) hNEIL1 luc. Starting with this plasmid, we generated several deletion constructs by PCR amplification, with sense primers representing different segments of the 5 regions of hNEIL1; the 5’ deletion fragments were subsequently fused upstream of the luciferase gene in the expression vector. The sequences of sense primers to generate these reporter plasmid constructs are given in Table 1. All of these were used in combination with a common antisense primer, 5’CTT CCC GGG TTC AAG TGA TTC TCC TGC CTC 3’. Point mutations were generated in p(-1800/+40)NEIL1 luc with the Quikchange Site-directed mutagenesis kit (Stratagene) per
manufacturer’s instructions. The following oligo sets were used as follows (mutated nucleotides are underlined): distal CRE/AP-1 site; 5′ GGCGAGGCTGCAGATCATGCTGAGGTTTTGAGACCAGC3′ and 5′GGCTGATCGATCTTC AAACCACCTCAGTCTGCTGCCTGCC3′; proximal CRE/AP-1 site; 5′GGCGAGGCTGCAGATCATGCTGAGGTTTTGAGACCAGC3′ and 5′GGCTGATCGATCTTC AAACCACCTCAGTCTGCTGCCTGCC3′. The nucleotide sequences of both deletion and mutant constructs were verified by sequencing. The HCT116 cells were transfected with the reporter plasmids using lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. The cells were harvested 36 h after transfection, and their extracts assayed for luciferase activity using a Dual-Luciferase Reporter Assay System (Promega) as per the manufacturer’s instructions. All experiments were performed at least in triplicate, and the data were normalized to co-transfected Renilla luciferase (control reporter) to correct for variation in transfection efficiency. Co-expression experiments were performed using expression vectors containing full-length cDNAs of the transcription factor c-Jun.

Statistical Analysis
In all experiments, including densitometric scans of Western blots or RT-PCR analysis, and luciferase assays, results were calculated as the means (+ S.D.) of three independent experiments. Statistical analyses were performed using one-way Anova for comparisons of multiple groups. P values of 0.05 or less were deemed to be statistically significant. All statistical tests were done using SigmaStat software (Jandel Scientific).

Results
Oxidative Stress Transiently Increases NEIL1 Protein and mRNA Levels in HCT116 Cells
To explore the effects of oxidative stress on NEIL1 expression, we treated HCT116 cells for 1 h with a sub-toxic dose (100 ng/ml) of the ROS generator GO, and then analyzed NEIL1 protein and mRNA levels at various times. GO treatment caused 2.4 ± 0.3-fold increase in the intracellular ROS level in these cells after 1 h (Fig. 1). Mock and GO-treated cells were harvested at 0, 3, 6, 9, 12 and 20 h. Western analysis of whole cell and more significantly nuclear extracts showed an increase in the NEIL1 protein level within 3 h of GO treatment, reaching up to 2- to 4-fold over the control at 6 to 9 h and before returning to the baseline by 20 h (Fig. 2). The OGG1 polypeptide level remained unchanged over a similar time course of GO treatment (Fig. 2A). The GO treatment similarly increased the NEIL1 mRNA level in HCT116 cells (up to 4 folds at 6-9 h), as analyzed by both semi-quantitative (Fig. 3A) and quantitative RT-PCR (Fig. 3B). The GO treatment was performed in parallel in MRC5, human primary fibroblasts. Real-time RT-PCR analysis of RNA revealed a similar induction of the transcript level upon exposure to ROS (Fig. 3B).

Identification and Characterization of cis Elements in the hNEIL1 Promoter
To further analyze the basis for ROS-induced activation of the NEIL1 gene, we characterized its cis elements. We cloned a 13 kb EcoRI/Hind III genomic fragment containing the complete transcription unit of the NEIL1 gene into pBluescript, together with a significant part of the 5′ flanking region. Comparison of the genomic sequences of hNEIL1 to its cDNA showed that the transcribed sequence spans approximately 8 kb, and contains 10 exons. The transcriptional start site (TSS) was determined by primer extension analysis; the longest product observed extended 40 bp from the primer, P2, indicating a G in the sense strand as the site most frequently used for transcription. (Fig. 4). Primer P1 failed to show any primer extension product. The initiation codon of the NEIL1 polypeptide was located in the 2nd exon, exon 1 (1 kb) being the untranslated region (UTR). Transfac software analysis of a 2.1 kb fragment spanning upstream sequences, including part of the 5′ exon identified several putative cis elements for transcription factors. It is interesting to note the presence of binding sites for several b-Zip family proteins, including c-Jun, CREB and NF-E2/AP-1-related factors (Fig. 5A).

To test for basal promoter activity of the upstream sequences, a NEIL1 promoter luciferase reporter construct p(-2100/+40)NEIL1 luc, containing NEIL1 genomic sequence -2100 to +40 bp, was used for transient expression in transfected HCT116 cells, as described in Experimental Procedures. A series of nested 5′ deletion mutants of the NEIL1 promoter were subsequently...
generated in pGL2-basic (as shown in Table 1 and Fig. 5B), and the luciferase activity measured. Deletion of the region between -1800 and -900 bp caused a 2- to 3-fold decrease of luciferase activity. Additional deletion of the downstream sequence to -600 bp or -500 bp did not further decrease promoter activity, whereas deletion of the region spanning -300 and -100 bp resulted in another 3-fold decrease in promoter activity, indicating that this region is the major contributor to NEIL1 expression. Furthermore, the minimal promoter activity could be detected in the plasmid containing the -50/+40 sequences.

Oxidative Stress Transactivates the NEIL1 Gene Promoter

To identify the promoter region essential for transcriptional upregulation of NEIL1 during oxidative stress, HCT116 cells were transfected with three different NEIL1 reporter constructs: p(-1200) NEIL1 luc (bearing both proximal and distal CRE/AP-1 elements); p(-900) NEIL1 luc (bearing only proximal CRE/AP-1); and p(-50) NEIL1 luc (lacking both CRE/AP-1 elements). The luciferase activity was measured in extracts of transfected cells after GO treatment (Fig. 6A). A >2-fold increase in luciferase activity was observed with the p(-1200) NEIL1 luc construct within 3 h after exposure to GO. This upregulation reached its peak at 6 h (> 3.5-fold) and returned to the basal level at 20 h. Similarly, a ~3-fold induction was observed with p (-900) NEIL1 luc after exposure to GO. However, the promoter-reporter construct p (-50) NEIL luc, containing the minimal promoter sequence, showed no upregulation in luciferase activity under these conditions. These results indicate that the cis-acting elements responsible for oxidative-stress induced NEIL1 promoter activity are located 60 to 1200 bp 5' to the transcriptional start site.

Oxidative Stress Activates the NEIL1 Promoter via a Pair of Proximal and Distal CRE/AP-1 Sequences

Examination of the sequence within the -60 to -1200 bp region of the NEIL1 promoter revealed two copies of putative elements at positions -61 and -1018, both with sequence similarity to the palindromic consensus CRE (TGACGTCA) and AP-1 (TGACTCA). To test whether these CRE-like sites are required for oxidative stress-induced expression of NEIL1, we mutated these sites either individually or simultaneously, and assayed the promoter activity, following GO treatment (Fig. 6B). Mutation of the proximal CRE (Mut 1), showed an ~ 4fold decrease in oxidative stress-induced promoter activation, while mutation of distal CRE/AP-1 (Mut 2), showed a 2-fold decrease compared to the wild type. Moreover mutation at both CRE/AP-1 sites (Mut 3) further decreased the GO-induced promoter activation by about ~ 6-fold. These observations suggest that the two CRE/AP-1 sites are independently involved in oxidative stress-mediated activation of the NEIL1 promoter.

Oxidative Stress Stimulates Binding of CREB/c-Jun to NEIL1 CRE/AP-1

To identify the transcription factors interacting with NEIL1-CRE/AP-1, we incubated nuclear extracts with 32P-labeled oligo duplexes of sequences corresponding to the proximal and distal NEIL1-CRE sites, respectively. As shown in Fig. 7, two closely spaced complexes were observed in both instances (indicated by arrows) that could be eliminated by addition of 100-fold molar excess of unlabeled NEIL1-specific or consensus AP-1 oligo. Lack of competition with a similar excess of mutated AP-1 oligos established the binding specificities of the proximal as well as distal NEIL1-CRE/AP-1 sequences. Similar results were obtained when the CRE consensus and mutant oligonucleotides were used as competitors in EMSA (data not shown). Additionally, no specific binding was observed with the mutated NEIL1-CRE/AP-1 oligo (last lane in panel i).

We then investigated whether oxidative stress leading to activation of the NEIL1 promoter was associated with increased binding of the CRE/AP-1 complex to the cis element. Treatment of HCT116 with GO resulted in a significant increase in the binding of the nuclear extract to the NEIL1 CRE/AP-1 (Fig. 7A and B, panel ii), as early as 30 min after treatment, which returned to the basal level within an hour. To identify the proteins present in the binding complexes, supershift analyses were performed with antibodies specific for various members of the CREB/ATF and AP-1 families (Fig. 7A and B, panel iii). Both anti-Jun (c-Jun/AP-1) and CREB-1 antibodies decreased the amount of the specific DNA-protein complexes. A pronounced supershifted complex was generated with the phospho-c-Jun antibody. Incubation of nuclear
extracts with ATF-1 antibody did not generate a supershifted band, while ATF-2 antibody slightly decreased the level of the specific complex. With extracts of stressed cells, the CRE complex was completely supershifted with addition of phosphorylated c-Jun-specific antibody, suggesting that under oxidative stress, the majority of c-Jun bound to the NEIL1-CRE was in the phosphorylated form. We have further concluded that NEIL1-CRE complex responsive to oxidative stress consists of c-Jun and CREB proteins.

**GO Treatment Stimulates Phosphorylation of c-Jun**

We then investigated whether oxidative insult by GO treatment, which induced NEIL1 promoter activity and CRE protein binding, increased the levels of CREB, c-Jun, and ATF2 polypeptides. Western blot analysis of GO-treated cells showed that the CREB and ATF2 levels were slightly enhanced, but a marked increase in the level of phosphorylated c-Jun was observed within 30 min exposure to GO (Fig. 8A). These results indicate that CRE/AP-1 activation was correlated with oxidative stress-induced upregulation of NEIL1 expression.

**c-Jun Induces Both Basal and Oxidative Stress Induced Promoter Activity of NEIL1**

The EMSA studies suggested that the oxidative stress-induced enhancement of the affinity of nuclear extracts for the NEIL1 promoter was due to activation of c-Jun. We directly tested this possibility by examining the effect of c-Jun overexpression on NEIL1 promoter activity. HCT116 cells were co-transfected with a c-Jun expression plasmid and p(-1800/+40)NEIL1 luc containing both copies of CRE/AP-1. c-Jun dose-dependent increase in luciferase activity was observed, and GO treatment further increased the activity by about 2-fold (Fig. 8B). Stimulation of activity due to c-Jun overexpression was significantly attenuated when both CRE/AP-1 were mutated in the NEIL1 promoter construct, Mut3 (Fig. 8B). This confirmed that promoter activation was mediated by the CREs.

**Discussion**

We observed oxidative stress-dependent activation of hNEIL using sublethal GO treatment for sustained ROS generation. An increase in the levels of both NEIL1 mRNA and polypeptide suggested that ROS enhances NEIL1 transcription. To elucidate the molecular basis of NEIL1 induction, we first characterized the hNEIL1 promoter. Functional analysis of nested deletions of the NEIL1 gene upstream of the coding region revealed that the basal promoter is localized in a 50 bp segment 5' to the transcription start site. However, robust promoter activity induced by the -1200 construct indicated the presence of major cis elements in the distal region of the promoter. Analysis of the sequence spanning -1800 to +40bp of the promoter suggested the presence of cis elements specific for various members of the bZip family of transcription factors. The b-Zip proteins are characterized by the presence of a basic domain required for interaction with DNA and an adjacent leucine zipper domain that allows dimerization between the family members (24). We identified a pair of nearly identical DNA sequences that differ by a single nucleotide from the palindromic consensus CRE, a target for binding of the bZip proteins CREB and ATF2 (25-27). Additionally, this CRE-like sequence is similar to the AP-1 element (28), and so could serve as a target for the binding of c-Jun and or c-Fos transcription factors, which also belong to the b-Zip family. To identify the specific trans-acting elements, we performed EMSA with a NEIL1 CRE oligo and nuclear extracts. Supershift of the specific complex with anti-c-Jun antibody, and decrease in binding of nuclear extract in the presence of anti-CREB and anti-ATF2 antibodies confirmed the presence of these proteins in the binding complex.

Further analysis of promoter reporter constructs containing both CRE/AP-1 sequences in the NEIL 5’ region showed a significant increase in luciferase activity relative to that with a deletion construct containing only the proximal CRE/AP-1. Oxidative stress activated both, while the minimal promoter lacking the CREs was not activated by oxidative stress. Mutational analysis further showed the requirement of key bases in both CRE/AP-1 for response to oxidative stress. These findings confirm that GO induces hNEIL1 expression via transactivation of the NEIL1 promoter. To the best of our knowledge, this is the first evidence for a role of CRE/AP-1 in NEIL1 expression.

In addition to their role in regulation of a variety of genes, the CRE/AP-1 family of transcription factors are among the most prominent regulators of the oxidative stress
response. Furthermore, the consensus sequence recognized by the classic CRE/AP-1 family members, 5′-TGAG/CTC/AA-3′, is similar to the antioxidant response element (ARE) core sequence, 5′-GTGACNNNGC-3′. The presence of similar sequences in the 5′ region of NEIL1 promoter strongly suggests that NEIL1 induction upon cellular exposure to ROS is regulated by binding of the members of the CREB/c-Jun family of transcription factors similar to these ARE-like upstream elements. Our studies also confirmed that c-Jun and the other related transcription factors are induced by GO treatment. Further studies will be needed to determine the mechanism by which GO promotes CREB/c-Jun binding in order to identify other members of the signaling pathway.

Several other mammalian repair genes are inducible by oxidative/genotoxic stress. The O6-methylguanine-DNA-methyltransferase (MGMT) gene is transcriptionally activated by different types of genotoxic agents, including alkylating drugs and ionizing radiation (29,30). The MGMT promoter contains two AP-1-binding sites, which play a key role in MGMT basal regulation (31). However, the elements required for MGMT induction remain to be identified. DNA polymerase β (Polβ) is induced by simple methylating agents, such as MNNG which is also mediated through a CREB (32). The Polβ expression level is also enhanced by oxidative treatments (33). Similarly expression of mammalian APE1 was induced by oxidative stress (34,35). The promoter of the human APE1 contains consensus sequences for binding NF-κB, CRE and AP-1. Similar to our observation for NEIL1, transcriptional activation of the APE1 gene was shown to require CREB/c-Jun (36). The human OGG1 promoter was recently shown to contain a pair of inverted CCAAT motifs that are crucial to its induction by the DNA-alkylating agent, MMS (37). Furthermore, the transcription factor NF-YA was shown to interact with the MMS regulatory elements. Earlier the yeast NTH1 gene was shown to contain multiple stress response elements in its promoter (38). However, we have shown here that OGG1 was not activated by oxidative stress. The teleological basis for activation of OGG1 and NTH1 by nonoxidants is not clear.

In summary, our results demonstrate that ROS upregulate hNEIL1 expression through activation of the transcription factors, CREB/c-Jun. An important question that remains to be answered is the biological role of hNEIL1 activation in response to oxidative stress. We hypothesize that endogenous ROS induce DNA damage at a basal, steady-state level in unstressed cells. However, exogenous oxidative stress, while increasing the genomic damage level, simultaneously activates the cellular response to repair the additional load of DNA damage. NEIL1 activation is a part of this coordinated cellular response. This scenario implies that NEIL1 or other proteins activated by oxidative stress are limiting in the repair pathway. It is possible that NEIL1 has a unique role among the oxidized base-specific mammalian DNA glycosylases, including OGG1 and NTH1, by preferentially repairing damage in active genes. Lack of OGG1 activation by ROS underscores the key difference in regulation of NEIL1 and OGG1. This is further supported by the observations that OGG1- or NTH1-null mouse fibroblasts are no more sensitive to ROS than the parental wild-type cells (12,39). In contrast, downregulation of NEIL1 expression by siRNA in mouse embryonic stem cells sensitizes these cells to γ-irradiation (40). Thus the fine tuning of NEIL1 expression, both in response to cell cycle or external oxidative stress, may be more critical than that of OGG1 or NTH1. How such modulation of NEIL1 expression is regulated in the context of global response remains to be investigated.

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Footnote:

Abbreviations

The following abbreviations are used: APE, AP endonuclease; AP-1, activator protein 1; ATF, activating transcription factor; CRE, cyclic AMP response element; EMSA, electrophoretic mobility shift analysis; 5-OHU, 5-hydroxyuracil; GO, glucose oxidase; Luc, luciferase; Mut, mutant; NE, nuclear extract; NEIL, Nei-like; NTH1, endonuclease III homolog 1; OGG1, 8-oxoguanine-DNA glycosylase 1; PBS, phosphate buffered saline; ROS, reactive oxygen species; WT, wild type.

Figure Legends

Figure 1: Changes in intracellular ROS levels after GO treatment. (A) HCT116 cells were mock-treated or treated with increasing amount of GO. Cells were subsequently loaded with 5 μM of H2DCF-DA and the change in DCF fluorescence was determined by flow cytometry as described in Experimental Procedures. (B) Kinetics of fluorescence induction in HCT116 cells exposed to 100 ng/ml GO.

Figure 2: Induction of hNEIL1 in oxidatively stressed HCT116 cells. (A) After treating HCT116 cells with GO (100 ng/ml), as described in Experimental Procedures, total lysates of cells, harvested at various times, were analyzed for NEIL1 and OGG1 levels by Western blotting; β-actin was used as the loading control (left panel); the ratios of band intensities of hNEIL1 or hOGG1 to β-actin are shown in the right panel. Results correspond to mean (± S.D.) from three separate experiments. (B) Left panel: Western analysis of nuclear extracts from GO-treated HCT116 cells; lamin B, loading control; right panel: Graphical representation of these results; *, p < 0.05; **, p < 0.01.

Figure 3: Activation of the hNEIL1 gene in response to oxidative stress. (A) Left panel: Quantitation of hNEIL1 mRNA in GO-treated HCT116 cells by semiquantitative RT-PCR as in Fig. 2. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal reference. Right panel: Graphical representation of hNEIL1 induction normalized to GAPDH. (B) Left panel: Quantitation of hNEIL-1 mRNA in MRC5 and HCT116 cells by real-time PCR, normalized to endogenous 18s RNA; right panel: Graphical representation of these results; p < 0.05.

Figure 4: Mapping of NEIL1 transcriptional initiation site. A pair of oligodeoxynucleotides, complementary to 5'-untranslated region (UTR) of the hNEIL1 cDNA, were annealed to 50 μg total RNA from HCT116 cells, and extended with AMV reverse transcriptase in separate reactions as described in Experimental Procedures. (A) Lane 1, primer extension product with P1 oligo; lane 2, product with P2 oligo. M, marker. (B) Dideoxynucleotide sequence of NEIL1 5'-flanking DNA region primed with P2 oligo showing C, T, A, and G ladders. The arrow indicates transcription initiation site (TSS), corresponding to the P2 primer extension product.

Figure 5: 5'-Upstream sequence of the hNEIL1 gene and characterization of its promoter activity. (A) The nucleotide sequence of the 5'-flanking region and part of the first exon of the hNEIL1 gene. The numbering is based on the transcriptional initiation site (+1). Consensus sequences for binding to the regulatory factors are shown, with highlighted CRE/AP-1 sequences. The arrows mark the complementary strand of the oligonucleotide used for primer extension with P2. (B) DNA fragments of the 5'-regulatory region were cloned upstream of luc coding sequence, and their promoter activity determined as described in Experimental Procedures; *, p < 0.05, **, p < 0.001.
Figure 6: Identification of CRE/AP-1-binding sites required for ROS-induced activation. (A) Luciferase activity after transfection with p(-1200) NEIL1 luc, p(-900) NEIL1 luc and p(-50) NEIL1 luc plasmids and treatment with GO (100 ng/ml). *, #, P < 0.05; **, ##, p < 0.001. (B) Effect of mutation of CRE/AP-1 at residues -61 and -1018 of NEIL1 promoter. WT, wild type, Mut1, mutation at the proximal CRE; Mut2, mutation at the distal CRE; Mut3 mutations at both CREs; Luc, pGL2-basic. Other details are given in Experimental Procedures; *, p < 0.05, **, p < 0.001.

Figure 7: Effects of ROS on the binding of cJun /CREB to CRE/AP-1 in the NEIL1 promoter. HCT116 nuclear extracts (2 µg) were incubated with 32P-labeled proximal (A) or distal (B) NEIL1 CRE/AP-1 oligo with molar excess of WT or mutant oligos for EMSA (panels i). I and II indicate specific complexes; (the nonspecific complex III was not competed with NEIL1 CRE/AP-1 or consensus CRE/AP-1 oligo). Binding experiments were performed with NEs of GO-treated cells (panel ii). For supershift analysis (panels iii), the reaction mixtures were incubated with antibodies to c-Jun, CREB, ATF1 and ATF2; the supershifted (ss) complexes are indicated by arrows.

Figure 8: Oxidative stress-induced increase in c-Jun /CREB levels. (A) Left panel NE (50 µg) of GO-treated HCT116 cells were used for Western analysis with antibodies against phospho c-Jun (KM1), CREB and ATF2. Lamin B was the loading control. Right Panel: Increase in CREB and ATF2 levels was quantified relative to the GO-untreated level. *, p < 0.05, **, p < 0.001. (B) HCT116 cells were co-transfected with WT NEIL1-luc (solid) or MutCRE NEIL1-luc (stripes) and c-Jun expression plasmid; other details are described in Experimental Procedures; *, p < 0.05, **, p < 0.001.
| Primer       | Primer Sequence          | Plasmid                   |
|-------------|--------------------------|---------------------------|
| 1800-luc sense | 5’GGT TAC CCA TGT TGT TGC ATG TGG CAG 3’ | p(-1800/+40) NEIL1 luc    |
| 1200-luc sense | 5’GAA CCA TAT GTT TTC TAA TGC AGA GG 3’ | p(-1200/+40) NEIL1 luc    |
| 900-luc sense  | 5’CTA CTC AGG AGG CTG AGG CAA GAG 3’  | p(-900/+40) NEIL1 luc     |
| 600-luc sense  | 5’ GTG ATG CAG CAT CTA TCT CAA ACA 3’ | p(-600/+40) NEIL1 luc     |
| 300-luc sense  | 5’ CTA CTT GGG AGG CTG AGG CTG AGG 3’ | p(-300/+40) NEIL1 luc     |
| 100-luc sense  | 5’ CTC ACT TCT GTA ATC CCA GCA CTT 3’ | p(-100/+40) NEIL1 luc     |
| 50-luc sense   | 5’GGT CAG GAG TTC AAGACC AGC CTG 3’  | p(-50/+40) NEIL1 luc      |
Fig 1. Das et al
Fig 2. Das et. al

A.

| GO  | 0   | 3   | 6   | 12  |
|-----|-----|-----|-----|-----|
| NEIL1 |     |     |     |     |
| OGG1  |     |     |     |     |
| β-actin |     |     |     |     |

B.

| GO  | 0   | 6   | 9   | 20  |
|-----|-----|-----|-----|-----|
| NEIL1 |     |     |     |     |
| Lamin B |     |     |     |     |
Fig 3. Das et al
Fig 4. Das et al.
Fig 5B. Das et. al
Fig 6A. Das et al.
Fig 6B. Das et al
Fig 7A. Das et al.
Fig 7B. Das et al.
Fig 8A. Das et al

[Image of Western blot analysis showing time points (GO, 0, 30, 120 minutes) and fold change for cJun (KM1), CREB, ATF2, and Lamin B proteins. Graph shows statistical significance markers (*, #, ##).]
Supplementary Figure
Induction of the human oxidized base-specific DNA glycosylase NEIL1 by reactive oxygen species
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