Oxidative stress-induced protein damage inhibits DNA repair and determines mutation risk and anticancer drug effectiveness

Elizabeth McAdam, **Reto Brem**, and **Peter Karran**[*]
The Francis Crick Institute, Clare Hall Laboratory, South Mimms EN6 3LD, UK

Abstract

The relationship between sun exposure and non-melanoma skin cancer risk is well established. Solar ultraviolet radiation (UV; wavelengths 280-400 nm) is firmly implicated in skin cancer development. Nucleotide excision repair (NER) protects against cancer by removing potentially mutagenic DNA lesions induced by UVB (280-320 nm). How the 20-fold more abundant UVA (320-400 mn) component of solar UV radiation increases skin cancer risk is not understood. We demonstrate here that the contribution of UVA to the effects of UV radiation on cultured human cells is largely independent of its ability to damage DNA. Instead, the effects of UVA reflect the induction of oxidative stress that causes extensive protein oxidation. Because NER proteins are among those damaged, UVA irradiation inhibits NER and increases the cells' susceptibility to mutation by UVB. NER inhibition is a common consequence of oxidative stress. Exposure to chemical oxidants, treatment with drugs that deplete cellular antioxidants, and interventions that interfere with glucose metabolism to disrupt the supply of cellular reducing power all inhibit NER. Tumor cells are often in a condition of oxidative stress and one effect of the NER inhibition that results from stress-induced protein oxidation is an increased sensitivity to the anticancer drug cisplatin.

Statement of implication: Since NER is both a defence against cancer a significant determinant of cell survival after treatment with anticancer drugs, its attenuation by protein damage under conditions of oxidative-stress has implications for both cancer risk and for the effectiveness of anticancer therapy.

Introduction

The ultraviolet radiation (UV) in sunlight causes skin cancer. Direct absorbance of UVB (280-320 nm) by DNA produces the cyclobutane pyrimidine dimers (CPDs) and pyrimidine 6:4 pyrimidone adducts (6:4 Py:Pys) that are the major DNA lesions induced by solar radiation (1). These lesions are responsible for the signature C to T changes at PyC sequences that dominate skin cancer mutation spectra (reviewed in (2)). The nucleotide excision repair (NER) system removes UVB-induced DNA photoproducts. This prevents mutation and protects against skin cancer. The impaired NER, extreme photosensitivity and skin cancer susceptibility in individuals with the genetic disorder xeroderma pigmentosum

[*]Corresponding author: peter.karran@crick.ac.uk.
The authors disclose no potential conflicts of interest

established the paradigm for the inverse relationship between effective DNA repair and cancer risk (3). Despite the protection afforded by NER, skin tumors (4–7) and even morphologically normal skin (8) accumulate extremely high numbers of UVB signature mutations. Surprisingly, mutation frequencies in non-melanoma skin cancers (NMSCs) in particular approach those in tumors in which disabled replication error-correcting systems confer extremely high spontaneous mutation rates (9). There are, however, no known deficiencies in DNA repair or editing in NMSCs or normal skin and it seems paradoxical that such large numbers of UVB-induced mutations coexist with an NER system dedicated to removing mutagenic DNA photolesions.

The contribution to cancer risk of UVA (wavelength 320-400 nm) that comprises 95% of incident solar UV radiation is enigmatic. Although UVA is classified as a human carcinogen by the WHO (10), it causes little direct DNA damage. UVA is, however, a source of reactive oxygen species (ROS) *via* interactions with cellular chromophores. DNA damage caused by UVA-induced ROS (11) is a potential contributor to sun-induced mutation and cancer. The signature mutations of oxidative DNA lesions are not, however, prominent in the mutational landscapes of skin tumors(12).

We have previously shown that some of the effects of UVA on cultured cells and skin are mimicked and amplified when UVA is combined with an exogenous UVA chromophore. Among these, the purine analog 6-thioguanine (a metabolite of the immunosuppressant azathioprine) and fluoroquinolone antibiotics are mixed Type I and Type II UVA photosensitizers that induce oxidative stress, an excess of ROS. The ROS generated in these photosensitized reactions cause widespread DNA damage. They also induce extensive protein oxidation (13, 14). One important consequence of this extensive oxidation is the inactivation of DNA repair by damage to essential proteins. These include the RPA single-strand DNA binding complex that is essential for NER (15) and cells treated with UVA/ photosensiitizer combinations have a reduced capacity to excise canonical UV photoproducts.

The extreme DNA damage resistance of certain bacteria (16) and microscopic aquatic animals (17) emphasizes the importance of preventing protein oxidation. These organisms deploy sophisticated antioxidant systems to maintain the integrity of cellular survival systems and protect them against the extreme oxidative stress that accompanies desiccation. The prevention of protein damage and maintenance of functional DNA repair is a major contributor to their enhanced resistance (18).

Several antioxidant systems protect human cells against oxidative stress. These forestall the potentially dangerous consequences of protein oxidation such as the formation of oxidized protein aggregates. Treatment with oxidants or changes in metabolism can perturb the balance between the formation and removal of ROS and induce oxidative stress. The altered metabolism of cancer cells means that many exist in conditions of sustained oxidative stress, a characteristic that is particularly associated with oncogenic transformation (19). Intracellular ROS levels can be an important determinant of the effectiveness of anticancer drugs. Pharmacological interventions to decrease antioxidant protection are generally associated with enhanced sensitivity to anticancer agents. It is noteworthy that many of these

kill tumor cells by inflicting potentially lethal DNA lesions that are substrates for DNA repair.

Although the contribution of oxidative DNA damage to cancer risk has been widely studied, relatively little attention has been paid to the possible influence of protein oxidation. The vulnerability of the DNA repair proteome to inactivation by oxidation by photosensitized UVA raises the possibility that DNA repair might be compromised under conditions of oxidative stress. In particular, that NER in skin cells might be partially inactivated by oxidative stress induced by solar UVA. In this report, we describe the impact of oxidative stress on DNA repair efficiency in cultured human keratinocytes. In particular, we show that DNA repair, including NER is inhibited by treatment with UVA radiation or oxidizing chemicals. Interventions that alter glucose metabolism and deplete antioxidant defences also inhibit NER providing further evidence for the involvement of oxidative stress. The vulnerability of DNA repair to inactivation by protein oxidation has implications for mechanisms of carcinogenesis and for the effectiveness of anticancer therapies.

Materials & Methods

Cell culture and treatments

Cell lines were obtained from Clare Hall Laboratory Cell Services. HaCaT (immortalised human keratinocyte) HeLa and HT1080 cells were cultured in Dulbecco's MEM, TK6 in RPMI. Media were supplemented with 10% foetal calf serum. All cell lines had been authenticated by STR profiling within the twelve months prior to use. Tests for mycoplasma contamination performed at the same time were negative. Buthionine-sulfoximine (BSO) treatment was 2 mM for 24 h prior to irradiation. H_2O_2 treatments were for 30 min. For D_2O treatment, cells were held for 30 min prior to irradiation in PBS/D_2O. Vitamin C treatments were for 3 hours prior to irradiation and cells cultured in vitamin C-containing medium during subsequent repair incubations. The IDH132 inhibitor AGI5198 was obtained from Millipore. To assay cell survival, treated cells were seeded into 6-well plates at a density of 300 cells/well and colonies stained and counted 10 days later.

UV irradiation

Cells were irradiated in PBS. UVA was delivered using a UVH 250 W iron bulb (UV Light Technology Limited, emission maximum 365 nm) at a dose rate of 0.1 kJ/m^2/s. UVB radiation (maximum 312 nm) was from a LF-215 60 W bulb (Uvitec Limited) at 5 J/m^2/s. 254 nm UVC was delivered by a Stratalinker UV Crosslinker (Stratagene) at 10 J/m^2/s.

ROS measurement

Cells were incubated in PBS containing CM-H_2CDFDA (Life Technologies) for 20 min at 37°C, irradiated and analysed by FACS.

Oxidised protein detection

Protein carbonyls—Cell extracts in RIPA buffer were incubated with 50 µg/ml Alexa Fluor 647 Hydroxylamine (FHA; Invitrogen) for 2 h at 37°C. Treated proteins (20 µg) were

separated on 10% Bis-Tris polyacrylamide gels (Invitrogen) and protein carbonyls visualised at 633 nm.

Protein sulfenates—Cells were lysed in RIPA buffer containing 1 mM biotin-1,3-cyclopentanedione (BP-1) probe (KeraFAST) and free thiols blocked by the immediate addition of 20 mM N-ethylmaleimide. Proteins were precipitated with acetone and redissolved in 2% SDS. Protein concentrations were determined by BCA assay (Thermo Scientific). Solubilized proteins were diluted 20-fold and mixed with pre-washed (3 x 4 volumes 0.1% SDS) M280 Streptavidin Dynabeads (Invitrogen). Following rotation overnight at 4°C, beads were washed sequentially with 2 M urea, 1 M NaCl, 0.1% SDS/10 mM DTT, and PBS (30 min each). Derivatized proteins were recovered by boiling and analysed by PAGE and immunoblotting.

TK mutation assay

Mutagenicity was measured by induction of trifluorothymidine (F_3TdR) resistant TK6 cells. Cultures were purged of pre-existing mutants by 48 h growth in HAT (100 μM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine; Sigma) medium followed by 24 h in HT (100 μM hypoxanthine, 16 μM thymidine). 24 h after return to normal medium, cells (0.5×10^5/ml in PBS) were irradiated, grown for 72 h and then seeded into 96-well plates at a density of 8000 cells/well in selective medium containing 2 μg/ml F_3TdR. Plating efficiency was assessed by seeding 1.6 cells/well in normal medium. Colonies were counted 10 days later. Mutation frequencies were calculated from the Poisson distribution and compared using the two-tailed Mann-Whitney test.

Photoproduct repair

Irradiated cells were returned to full medium at 37°C and sampled at different times. DNA was extracted using the QIAamp DNA mini kit (Qiagen) and 6:4 Py:Pys and CPDs measured by ELISA (Cosmo Bio). Data from all 6:4 Py:Py ELISAs of cells treated with either 200 J/m^2 UVB or 200 kJ/m^2 UVA + 200 J/m^2 UVB were pooled to create historical values (9 and 6 replicates, respectively). Graphical data points represent the mean of at least two independent experiments ± standard deviation.

In vitro NER

Dual-incision assays were performed according to the modified(13) method of Laine *et al*(49).

Immunoblotting

Cell extracts (20 μg) in RIPA buffer were separated on either 3–8% Tris-Acetate (MCM2) or 10% Bis-Tris gels (PCNA, RPA32, global protein sulfenates) (Invitrogen), and transferred to Immobilon-P membranes (Millipore). Antibodies used were from Santa Cruz (MCM2, PCNA) and Abcam (RPA32, G6PD). Streptavidin was from Invitrogen. Binding was detected by ECL reagent (GE Healthcare).

NADPH measurements

NADPH was measured using the NADP/NADPH Assay kit (abcam) according to the manufacturer's instruction.

RNA interference

siRNAs against G6PD were On-target smart pools from Dharmacon and were transfected using RNAiMax (Invitrogen) according to the manufacturer's protocol. Cells were analyzed 72 h after transfection.

Results

UVA (but not UVB or UVC) generates ROS and inhibits DNA repair

UVA irradiation induces ROS in human cells. Figure 1A shows that exposure of HaCaT cells to 100 or 200 kJ/m^2 UVA generated ROS and that this caused a measurable increase in the levels of both protein carbonyls and cysteine sulfenates that are acknowledged markers of oxidative protein damage (Figure 1B,C). UVB and UVC were much less effective in generating ROS even at doses that induced approximately 10-times more canonical UV lesions as determined by ELISA. Consistent with lower levels of ROS induction, neither UVB nor UVC detectably increased protein carbonylation. The predominant DNA photoproduct of UVA radiation is the T<>T CPD (11) but the formation of these photoproducts does not account for UVA cytotoxicity. At equivalent initial CPD levels, UVA is at least 5-fold more cytotoxic in HaCaT cells than UVC or UVB (Supplementary Figure 1). This observation is consistent with earlier suggestions that the cytotoxicity of longer UV wavelengths is not simply related to the induction of CPDs in human fibroblasts (20).

Since the initial photoproduct levels cannot account for UVA toxicity in HaCaT cells, we examined whether UVA affected the persistence of potentially lethal DNA lesions by interfering with NER. To analyse the efficiency of NER, we measured the rate of disappearance of 6:4 Py:Py photoproducts induced by UVB. These DNA-distorting lesions are excised promptly from human cells with a half life of 2-4 h. Consistent with this rapid repair, ELISA measurements indicated that four hours after irradiation with 200 J/m^2 UVB, HaCaT cells had excised approximately 70% of the initial 6:4 Py:Py photoproducts (Figure 1D). When HaCaT cells were also exposed to UVA which does not induce these lesions (11), the removal of 6:4 Py:Py photoproducts was impaired and the rate of their repair was reduced in a UVA dose-dependent manner. At 200 kJ/m^2 UVA, the rate of repair was less than 50% of that of cells that had not been exposed to UVA. Thus, NER is inhibited by UVA doses that induce detectable protein oxidation.

UVA irradiation also compromised the removal of CPDs, the more abundant photoproducts induced by UVB. UVA, UVB and UVC all induce CPDs albeit with widely different efficiencies. To ensure an appropriate comparison, HaCaT cells were exposed to doses of UVC, UVB and UVA that induced similar initial numbers of CPDs. Compared to 6:4 Py:Pys, these photoproducts are repaired more slowly by NER and ELISA measurements indicated that 24 h after irradiation, HaCaT cells had removed approximately 70% of the initial CPDs induced by 7 J/m^2 UVC or 300 J/m^2 UVB (Figure 1E). This figure is consistent

with the reported half-life of CPDs in NER-proficient human keratinocytes (21). In contrast, 24 h after irradiation with 300 kJ/m^2 UVA, more than 50% of CPDs remained in HaCaT DNA (Figure 1E). This observation is consistent with previous reports that human keratinocytes excise UVA-induced T<>T CPDs more slowly than the same lesion induced by UVB (22, 23). Taken together, the extended persistence of UVA-induced 6:4 Py:Pys and CPDs indicates that exposure to UVA compromises NER in HaCaT cells.

UVA also inhibited base excision repair (BER) of an oxidative DNA lesion. HaCaT cells were treated with KBrO$_3$ to induce DNA 8-oxoG, a substrate for removal by BER. ELISA measurements indicated that more than 60% of the initial DNA 8-oxoG was excised within 20-30 minutes. When KBrO$_3$ treatment was preceded by irradiation with 200 kJ/m^2 UVA, HaCaT cells removed less than 25% of the initial DNA 8-oxoG in 30 min (Figure 1F). The slower repair was not due to an increased DNA 8-oxoG level in irradiated cells. 8-oxoG was not detectable by ELISA following treatment with 200 kJ/m^2 UVA. This observation is in agreement with reports that UVA induces relatively few DNA 8-oxoG lesions (< 0.1% of T<>T) (11).

Oxidant treatment inhibits DNA repair

To examine whether NER inhibition was due to UVA-induced oxidative stress, we treated HaCaT cells with the oxidizing agent hydrogen peroxide. Although H$_2$O$_2$ is unreactive, it is converted to ROS *via* the Fenton reaction and causes DNA and protein damage. H$_2$O$_2$ induced protein carbonylation (Figure 1G) and inhibited the NER of UVB-induced DNA photoproducts by HaCaT keratinocytes. ELISA measurements indicated that four hours after UVB irradiation, H$_2$O$_2$-treated cells had only excised around 40% of 6:4 Py:Pys, compared to 65% by untreated cells (Figure 1H). Thus, UVA and H$_2$O$_2$, both of which induce oxidative stress and increase protein oxidation in HaCaT cells inhibit DNA repair.

UVA sensitizes cells to UVB mutagenesis

Because NER provides the main protection against mutation by solar UVB, we examined whether NER inhibition by UVA detectably affected UVB-induced mutagenesis. Figure 2 shows that UVA and UVB are synergistically mutagenic in the standard TK6 mutation assay (24). Initial measurements determined that 2 J/m^2 UVB approximately doubled the background frequency of $(3.8 \pm 1.6) \times 10^{-6}$ trifluorothymidine resistant (F$_3$TdR^R) TK6 mutants. Cells were then irradiated with 2 J/m^2 UVB combined with 20 or 50 kJ/m^2 UVA and the mutant frequency was compared to the sum of the frequencies induced by the same doses of UVB or UVA alone. A combination of 2 J/m^2 UVB with 20 kJ/m^2 UVA induced $(25.0 \pm 11.8) \times 10^{-6}$ F$_3$TdR^R mutants. This value is more than two-fold (p = 0.041) higher than $(10.6 \pm 14.1) \times 10^{-6}$ which is the sum of the frequencies $(3.6 \times 10^{-6} + 7.0 \times 10^{-6})$ induced by 2 J/m^2 UVB and 20 kJ/m^2 UVA, respectively. When cells were irradiated with a combination of 2 J/m^2 UVB and 50 kJ/m^2 UVA, the F$_3$TdR^R frequency increased to $(53.7 \pm 29.2) \times 10^{-6}$. This value is again significantly (p = 0.002) higher than $(19.1 \pm 7.4) \times 10^{-6}$ which is the aggregate frequency for 2 J/m^2 UVB and 50 kJ/m^2 UVA $(3.6 \times 10^{-6} + 15.5 \times 10^{-6})$.

These findings indicate that the risk of mutation by combined UVA and UVB is higher than from the same doses delivered separately. They are consistent with UVA-mediated attenuation of NER and the persistence of potentially mutagenic DNA photoproducts.

Protein damage is responsible for DNA repair inhibition

To assess directly the possible involvement of protein damage in UVA-induced inhibition of DNA repair, we determined the ability of extracts from irradiated cells to perform NER *in vitro.* These experiments were carried out using HeLa cells. Preliminary measurements by ELISA confirmed that UVA inhibited 6:4 Py:Py excision by HeLa cells *in vivo* and that the extent of inhibition was comparable to that in HaCaT cells (Supplementary Figure S2).

Extracts prepared from UVA irradiated HeLa cells were less proficient in excising a damage-containing oligonucleotide from a platinated plasmid in a standard NER assay (13) (Figure 3A upper panel) and this effect was UVA dose-dependent. Neither UVB nor UVC affected *in vitro* NER. Excision by extracts from cells that had been irradiated with UVB and UVC ($200 J/m^2$; $20 J/m^2$) to induce high levels of DNA photoproducts but no measurable protein oxidation, was comparable to that by extracts from unirradiated cells (Figure 3A lower panel). The values from three independent assays are shown in Figure 3B. Quantitation indicated that $200 kJ/m^2$ UVA inhibited NER by more than 50%. The impaired NER activity in extracts of UVA-treated cells provides unequivocal evidence that protein damage underlies the attenuation of NER by UVA.

We previously showed that the RPA DNA binding complex is limiting for NER. RPA is particularly susceptible to inactivation by oxidation *in vitro* (25) and *in vivo* (15). Since RPA thiol groups are particularly susceptible to inactivating oxidation (25), we probed extracts of UVA-irradiated HeLa cells for oxidized thiols in RPA. Figure 3C shows that increased oxidation of the NER-limiting RPA complex was detectable in extracts of HeLa cells that had received doses of UVA that inhibited NER.

Proteins are particularly susceptible to oxidation by singlet oxygen ($^1O_2$) (26). UVA is a significant inducer of intracellular $^1O_2$. The longevity and reactivity of $^1O_2$ is increased in D_2O. Consistent with an enhanced reactivity with cellular proteins, protein carbonyl levels were higher when HaCaT cells were UVA irradiated in D_2O (Figure 3D). The increased protein oxidation was accompanied by significantly enhanced NER inhibition and irradiation in D_2O with $200 kJ/m^2$ UVA effectively abolished 6:4 Py:Py excision by HaCaT cells (Figure 3E). These observations provide further evidence for the involvement of protein oxidation in NER inhibition and indicate that $^1O_2$ contributes to NER impairment by UVA in HaCaT cells.

Enhanced oxidative stress increases UVA-mediated DNA repair inhibition

The relationship between oxidative stress and NER inhibition was probed further by directly depleting antioxidant defences to increase steady-state ROS levels. The antioxidant glutathione (GSH) is essential for redox homeostasis. As expected, inhibition of GSH synthesis by treatment of HaCaT cells with the glutamylcysteine synthetase inhibitor BSO decreased the levels of cellular GSH by at least 99%. The oxidative stress associated with BSO treatment elevated the steady-state protein carbonylation level and this was further

increased by UVA irradiation (Figure 4A). Immunoblotting confirmed the increased protein damage and revealed that BSO treatment increased intersubunit crosslinking in the PCNA and MCM protein complexes, additional markers of oxidative protein damage (Figure 4B). Measurements of 6:4 Py:Py removal indicated that BSO treatment significantly potentiated NER inhibition by UVA. Four hours after irradiation with 200 kJ/m^2 UVA, more than 90% of UVB-induced 6:4 Py:Py photoproducts remained in DNA of BSO-treated HaCaT cells (Figure 4C).

Ascorbate (vitamin C) can have pro-oxidant effects and induce high levels of H_2O_2 that cause oxidative stress (27). High, pharmacological concentrations of ascorbate induce severe oxidative stress, toxicity and oxidative DNA damage in cultured ovarian carcinoma cells. Ascorbate-induced oxidative stress is associated with a sensitization of ovarian carcinoma cells to killing by carboplatin in culture and in xenografts (28). Treatment of HaCaT cells with high ascorbate concentrations induced oxidative stress and depleted NADPH levels (Figure 4D). NADPH is essential for the regeneration of reduced GSH and the maintenance of redox homeostasis. Ascorbate treatment of HaCaT cells induced a dose-dependent increase in protein carbonylation and attenuated the excision of UVB-induced 6:4 Py:Pys by NER (Figure 4E,F). The inhibitory effects of ascorbate were additive with those of UVA and the combination of high ascorbate concentration with 150 kJ/m^2 UVA was sufficient to almost completely inhibit NER (Figure 4F).

NADPH is essential for maintaining redox homeostasis and cells respond to increased oxidative stress by diverting glucose metabolism through the pentose phosphate pathway (PPP) that provides a major source of NADPH. Silencing of G6PD (Figure 5A), the first and rate-limiting step of the PPP depleted HaCaT NADPH levels by approximately 50%. Treatment with UVA (200 kJ/m^2) also depressed NADPH levels and the effect of irradiation was additive with that of G6PD silencing (Figure 5B). G6PD silencing also enhanced protein carbonylation by UVA (Figure 5C). It inhibited NER and potentiated UVA-induced NER inhibition. Four hours after irradiation with 200 kJ/m^2 UVA and UVB, G6PD silenced cells had excised less than 10% of the initial 6:4 Py:Pys (Figure 5D).

To investigate further the connection between NADPH levels and NER inhibition, we used HT1080 chondrosarcoma cells. These cells are heterozygous for a R132H mutation in the IDH1 isoform of isocitrate dehydrogenase (IDH) that catalyses the oxidative decarboxylation of isocitrate to α-ketoglutarate in the TCA cycle. This reaction also generates NADPH (30, 31). The gain of function R132H mutation enables the reduction of α-ketoglutarate to α-hydroxyglutarate in a reaction that consumes NADPH and compromises antioxidant protection. The HT1080 IDH1 inhibitor AGI5198 specifically targets the mutated enzyme to prevent NADPH depletion (32). As expected, treatment of HT1080 cells with AGI5198 boosted their NADPH level. Increased NADPH levels provided protection against the effect of UVA in HaCaT cells and AGI5198 treatment reversed both the NADPH depletion and the NER inhibition induced by UVA (Supplementary Figure 3).

Cisplatin is a member of the platinum-based family of drugs that is particularly effective against testicular and ovarian carcinomas. The toxicity of cisplatin is largely dependent on the induction of DNA damage and it is well established that NER significantly attenuates the

therapeutic effectiveness of platinum drugs by removing potentially toxic platinum-DNA lesions (29). Figure 5E shows that G6PD silencing sensitized HaCaT cells to killing by cisplatin. The enhanced cisplatin sensitivity in G6PD-silenced HaCaT cells is consistent with a significant reduction in NER activity by the oxidative stress induced by inhibiting the PPP.

Our experiments identify several interventions that cause inhibition of DNA repair. Although each of the treatments may have additional effects, they all share the ability to induce oxidative stress and to measurably increase protein oxidation. Taken together our observations reveal a significant susceptibility of DNA repair to inhibition under oxidative stress conditions. Inhibition reflects damage to members of the DNA repair proteome. The impact on NER of quite modest UVA doses is additive with that of antioxidant depletion.

Discussion

DNA repair pathways remove potentially mutagenic and lethal DNA lesions produced by radiation or chemicals. In doing so they provide protection not only against the initiation of carcinogenesis but also against the intended toxicity of many anticancer treatments. The sensitivity of DNA repair to inactivation by oxidative stress has significant implications for both cancer development and cancer treatment.

UVA radiation is a significant source of oxidative stress in human cells and both DNA and proteins are major targets for oxidation damage. UVA compromised the BER of the common DNA oxidation product, 8-oxoG in HaCaT cells. The OGG-1 and MUTYH DNA glycosylases that are essential participants in the removal of DNA 8-oxoG by BER, are known to be susceptible to inhibition by oxidation (13, 33). The common S326C-OGG-1 variant that is particularly prone to oxidation (33) is associated with increased risk of several forms of cancer (34). The presence of an essential iron-sulfur cluster in MUTYH (35) may explain its oxidation sensitivity. We have previously shown that oxidative stress resulting from the interaction between UVA and photosensitizing drugs also inhibits the non-homologous end-joining (NHEJ) pathway of DNA double-strand break (DSB) repair (13). In that case, oxidation damage to the essential dimeric Ku DNA end-binding complex is responsible for repair inhibition. Damage to Ku also occurs during oxidative stress induced by G6PD silencing and this too is associated with NHEJ inhibition (36, 37). All of these observations support our conclusion that damage to DNA repair proteins and inhibition of DNA repair is a general consequence of oxidative stress. Somewhat surprisingly, the repair of DNA lesions induced directly by ROS (DNA 8-oxoG) or as a secondary consequence of ROS-induced DNA damage (DSBs) can itself be compromised by oxidation damage to DNA repair proteins. The vulnerability of NER to damage by UVA is, however, particularly significant as it has implications for the mechanism by which solar radiation induces skin cancer.

The NER pathway, the major protection against skin cancer (1), can be inhibited in HaCaT keratinocytes by treatment with oxidizing chemicals, by UVA radiation and by treatments that deplete antioxidant defences. UVA is very poorly absorbed by DNA and induces little DNA damage. The predominant UVA photoproduct is the T<>T CPD, whereas oxidized

DNA bases are generated much more infrequently (11). Despite the T<>T CPD being the major photoproduct of both UVB and UVA, mutations at TT dinucleotides are not common in skin tumors. This presumably reflects error-free processing of this lesion by DNA polymerase η (38). It has been clear for some time that the production of CPDs cannot fully account for the severity of the cellular effects of UVA and there is no consistent indication of a significant contribution from oxidative DNA lesions. Overall, the cellular responses to UVA point to the existence of additional damage targets. Our findings reveal that the proteome is a biologically significant alternative target and that NER is inhibited by UVA-induced protein damage.

There is longstanding, albeit indirect evidence that UVA negatively affects NER. UVC-induced unscheduled DNA synthesis (UDS, a measure of the late, gap-filling step of NER) is reduced by UVA irradiation (365 nm) of human fibroblasts or lymphocytes (39, 40). Moreover, simulated solar radiation that contains both UVB and UVA was reported to be less effective than UVB alone in stimulating UDS (41). Experiments with bacteria provided more direct evidence that UVA impairs NER. UVA (365 nm) irradiation sensitized *E. coli* to killing by UVC (254 nm) and this increased sensitivity was associated with the prolonged persistence of CPDs (42). Our findings indicate that a similar NER inhibition is most likely responsible for many of the reported effects of UVA on human cells. We can now ascribe the inhibitory effects of on UVA on NER to ROS-induced protein damage. Solar UV to which skin is exposed contains a 20-fold excess of UVA over UVB. UVA and UVB were synergistically mutagenic in our experiments. Sequencing studies of morphologically normal skin and NMSCs from genetically NER-competent individuals have revealed huge numbers of mutations which bear the signature of UVB-induced DNA damage (8). These studies confirm that solar UVB is a powerful mutagen/carcinogen. They also indicate that mutations arise overwhelmingly at sites that have escaped repair by NER. In complete contrast, UVB phototherapy, which may involve > 100 exposures to DNA damaging doses of UVB is not associated with an increased skin cancer risk (43–45). It appears that therapeutic UVB is significantly less mutagenic/carcinogenic than solar UVB. Based on our findings, we suggest that this surprising discrepancy reflects the contribution of solar UVA. A NER system that is slightly compromised by the oxidative stress induced by coincident UVA would allow UVB mutations to accumulate. Chronic, low-level attenuation of NER by UVA-induced protein damage in sun-exposed skin might go some way to explaining the apparently paradoxical coexistence of high levels of sunlight-associated mutations with an NER system dedicated to the removal of potentially mutagenic UVB-induced DNA damage.

Our findings demonstrate that the level of oxidative stress determines the effectiveness of DNA repair. The efficiency of NER was compromised by oxidative stress induced by chemical oxidants and UVA radiation as well as by depletion of cellular antioxidant levels. Sustained oxidative stress is a common property of cancer cells. Although attempts to exploit this by further increasing ROS levels to breach a toxic threshold have met with mixed success (46), recent evidence indicates that treatment with ascorbate may be successful in this regard. At high concentrations, ascorbate generates H_2O_2 that causes widespread oxidative damage *via* the Fenton reaction (27). Several studies have indicated that these pharmacological ascorbate concentrations can potentiate the toxicity of ionizing radiation and anticancer drugs that induce DNA damage (28, 47). In our experiments, one

consequence of exposure of HaCaT cells to high ascorbate concentrations was NER inhibition by protein oxidation. The effect of ascorbate on NER was additive with that of UVA. Supplementing conventional carboplatin-based chemotherapy regimes with ascorbate increases DNA damage levels and toxicity in cultured ovarian tumor cells and improves the response to carboplatin-based therapy in ovarian tumor xenografts and in ovarian cancer patients (28). Carboplatin and the closely related cisplatin are members of the platinum family of DNA damaging anticancer drugs. A significant part of their toxicity is due to the induction of potentially lethal intrastrand DNA crosslinks. These lesions are good substrates for removal by NER and NER status is an important determinant of the effectiveness of platinum-based therapy. The intrinsically low NER capacity of testicular carcinoma cells (48) is a significant contributor to the spectacular success of platinum therapy against these tumors. In our experiments, high concentrations of ascorbate inhibited NER in HaCaT cells and it seems likely that a part of the ascorbate-mediated improvement in the responsiveness of ovarian tumors to carboplatin-based therapy reflects a similar NER inhibition. Consistent with this possibility, we found that the increased oxidative stress that accompanies G6PD silencing also increased the sensitivity of HaCaT cells to cisplatin.

Conjugation to GSH is a common mechanism of drug detoxification that depletes antioxidant protection and can lead to oxidative stress. Cisplatin is one example of the many chemicals, including numerous anticancer drugs that both induce oxidative stress and inflict DNA damage. Additional protein oxidation-related NER inhibition by adjuvant treatment may further improve the therapeutic effect of these anticancer agents. The high burden of UVB signature mutations in NMSCs suggests that NER might be compromised by UVA. It is noteworthy in this regard that the signature mutations in many other tumors clearly reflect unrepaired DNA lesions (12) and NER attenuation by oxidative stress is a possible contributor to their development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

References

1. Friedberg, EC.; Walker, GC.; Siede, W.; Wood, RD.; Schultz, RA.; Ellenberger, T. DNA Repair and Mutagenesis. Second edition. Washington: ASM Press; 2006.

2. Brash DE. UV signature mutations. Photochem Photobiol. 2015; 91:15–26. [PubMed: 25354245]

3. Cleaver JE. Xeroderma pigmentosum: a human disease in which an initial stage of DNA repair is defective. Proc Natl Acad Sci USA. 1969; 63:428–435. [PubMed: 5257133]

4. Durinck S, Ho C, Wang NJ, Liao W, Jakkula LR, Collisson EA, et al. Temporal dissection of tumorigenesis in primary cancers. Cancer Discovery. 2011; 1:137–143. [PubMed: 21984974]

5. Jayaraman SS, Rayhan DJ, Hazany S, Kolodney MS. Mutational landscape of basal cell carcinomas by whole-exome sequencing. J Invest Dermatol. 2014; 134:213–220. [PubMed: 23774526]

6. South AP, Purdie KJ, Watt SA, Haldenby S, den Breems N, Dimon M, et al. *NOTCH1* mutations occor early during cutaneous squamous cell carcinogenesis. J Invest Dermatol. 2014; 34:2630–2638. [PubMed: 24662767]

7. Harms PW, Vats P, Veraegen ME, Robinson DR, Wu Y-M, Dhanasekaran SM, et al. The distinctive mutational spectra of polyoma-negative merkel cell carcinoma. Cancer Res. 2015; 75:3720–3727. [PubMed: 26238782]

8. Martincorena I, Roshan A, Gerstung M, Ellis P, Loo PV, McLaren S, et al. High burden and pervasive positive selection of somatic mutations in normal human skin. Science. 2015; 348:880–885. [PubMed: 25999502]

9. Vogelstein B, Papadopoulos N, Velculescu VE, Zhou S, Diaz LA, Kinzler KW. Cancer genome landscapes. Science. 2013; 339:1546–1558. [PubMed: 23539594]

10. (IARC). Solar and ultraviolet radiation. IARC Monographs. 2010

11. Cadet J, Douki T, Ravanat J-L. Oxidatively generated damage to cellular DNA by UVB and UVA. Photochem Photobiol. 2015; 91:140–155. [PubMed: 25327445]

12. Alexandrov LB, Nik-Zainal S, Wedge DC, Aparicio SA, Behjati S, Biankin AV, et al. Signatures of mutational processes in human cancer. Nature. 2013; 500:415–421. [PubMed: 23945592]

13. Gueranger Q, Li F, Peacock M, Larnicol-Fery A, Brem R, Macpherson P, et al. Protein oxidation and DNA repair Inhibition by 6-thioguanine and UVA radiation. J Invest Dermatol. 2014; 134:1408–1417. [PubMed: 24284422]

14. Peacock M, Brem R, Macpherson P, Karran P. DNA repair inhibition by UVA photoactivated fluoroquinolones and vemurafenib. Nucleic Acids Res. 2014; 42:13714–13722. [PubMed: 25414333]

15. Guven M, Brem R, Macpherson P, Peacock M, Karran P. Oxidative damage to RPA limits the nucleotide excision repair capacity of human cells. J Invest Dermatol. 2015; 135:2834–2841. [PubMed: 26134950]

16. Slade D, Radman M. Oxidative stress resistance in *Deinococcus radiodurans*. Microbiology and Molecular Biology Reviews. 2011; 75:133–191. [PubMed: 21372322]

17. Krisko A, Leroy M, Radman M, Meselson M. Extreme anti-oxidant protection against ionizing radiation in bdelloid rotifers. Proc Natl Acad Sci USA. 2011; 109:2354–2357. [PubMed: 22308443]

18. Krisko A, Radman M. Phenotypic and genetic consequences of protein damage. PLoS Genetics. 2013; 9:e1003801. [PubMed: 24068966]

19. Sabharwal SS, Schumacker PT. Mitochondrial ROS in cancer:initiators, amplifiers or an Achilles' heel. Nat Rev Cancer. 2014; 14:709–721. [PubMed: 25342630]

20. Enninga IC, Groenendijk RTL, Filon AR, van Zeeland AA, Simons JWIM. The wavelength dependence of UV-induced pyrimidine dimer formation, cell killing and mutation induction in human diploid fibroblasts. Carcinogenesis. 1986; 7:1829–1836. [PubMed: 3769130]

21. Ferguson BE, Oh DH. Proficient global nucleotide excision repair in human keratinocytes but not fibroblasts deficient in p53. Cancer Res. 2005; 65:8723–8729. [PubMed: 16204041]

22. Courdavault S, Baudouin C, Charveron M, Canguilhem B, Favier A, Cadet J, et al. Repair of the three main types of bipyrimidine DNA photoproducts in human keratinocytes exposed to UVB and UVA radiations. DNA Repair. 2005; 4:836–844. [PubMed: 15950551]

23. Mouret S, Baudouin C, Chaveron M, Favier A, Cadet J, Douki T. Cyclobutane pyrimidine dimers are predominant DNA lesions in whole skin exposed to UVA radiation. Proc Natl Acad Sci USA. 2006; 103:13765–13770. [PubMed: 16954188]

24. Liber HL, Thilly WG. Mutation assay at the thymidine kinase locus in diploid human lymphoblasts. Mutat Res. 1982; 94:467–485. [PubMed: 6810168]

25. Men L, Roginskaya M, Zou Y, Wang Y. Redox-dependent formation of disulfide bonds in replication protein A. Rapid Commun Mass Spectrometry. 2007; 21:2743–2749.

26. Davies MJ. Singlet oxygen-mediated damage to proteins and its consequences. Biochem Biophys Res Comm. 2003; 305:761–770. [PubMed: 12763058]

27. Du J, Cullen JJ, Buettner GR. Ascorbic acid: Chemistry, biology and the treatment of cancer. Biochim Biophys Acta. 2012; 1826:443–457. [PubMed: 22728050]

28. Ma Y, Chapman J, Levine M, Polireddy K, Drisko J, Chen Q. High-dose parenteral ascorbate enhanced chemosensitivity of ovarian cancer and reduced toxicity of chemotherapy. Sci Transl Med. 2015; 6(222):222ra18.

29. Kelland L. The resurgence of platinum-based cancer chemotherapy. Nat Rev Cancer. 2007; 7:573–584. [PubMed: 17625587]

30. Jin G, Pirozzi CJ, Chen LH, Lopez GY, Duncan CG, Feng J, et al. Mutant IDH1 is required for IDH1 mutated tumor cell growth. Oncotarget. 2012; 3:774–782. [PubMed: 22885298]

31. Cairns RA, Mak TW. Oncogenic isocitrate dehydrogenase mutations: mechanisms, models, and clinical opportunities. Cancer Discov. 2013; 3:730–741. [PubMed: 23796461]

32. Molenaar RJ, Botman D, Smits MA, Hira VV, van Lith SA, Stap J, et al. Radioprotection of IDH1-mutated cancer cells by the IDH1-mutant inhibitor AGI-5198. Cancer Res. 2015; 75:4790–4802. [PubMed: 26363012]

33. Bravard A, Vacher M, Moritz E, Vaslin L, Hall J, Epe B, et al. Oxidation status of human OGG1-S326C polymorphic variant determines cellular DNA repair capacity. Cancer Res. 2009; 69:3642–3649. [PubMed: 19351836]

34. Karahalil B, Bohr VA, Wilson DM. Impact of DNA polymorphisms in key DNA base excision repair proteins on cancer risk. Hum Exp Toxicol. 2012; 31:981–1005. [PubMed: 23023028]

35. Brinkmeyer MK, David SS. Distinct functional consequences of MUTYH variants associated with colorectal cancer: damaged DNA affinity, glycosylase activity and interacton with PCNA and Hus1. DNA Repair. 2015; 34:39–51. [PubMed: 26377631]

36. Ayene IS, Stamato TD, Mauldin SK, Biaglow JE, Tuttle SW, Jenkins SF, et al. Mutation in the glucose-6-phosphate dehydrogenase gene leads to inactivation of Ku DNA end binding during oxidative stress. J Biol Chem. 2002; 277:9929–9935. [PubMed: 11788599]

37. Cosentino C, Grieco D, Costanzo V. ATM activates the pentose phosphate pathway promoting anti-oxidant defence and DNA repair. EMBO J. 2011; 30:546–555. [PubMed: 21157431]

38. Yagi Y, Ogawara D, Iwai S, Hanaoka F, Maki H. DNA polymerase eta and kappa are responsible for error-free translesion synthesis activity over a cis-syn thymine dimer in Xenopus laevis extracts. DNA Repair. 2005; 4:1252–1269. [PubMed: 16055392]

39. Smith PJ, Paterson MC. Lethality and the induction and repair of DNA damage by far, mid or near UV-irradiated human fibroblasts: comparison of the effects in normal, xeroderma pigmentosum and Bloom's syndrome cells. Photochem Photobiol. 1982; 36:333–343. [PubMed: 7146109]

40. Holmberg M. Prior exposure of human cells to near UV radiation gives a decrease in the amount of the unscheduled DNA synthesis induced by far UV radiation. Photochem & Photobiol. 1983; 37:293–295.

41. Parsons PG, Hayward IP. Inhibition of DNA repair synthesis by sunlight. Photochem Photobiol. 1985; 42:287–293. [PubMed: 4059365]

42. Tyrrell RM, Webb RB. Reduced dimer excision in bacteria following near ultraviolet (365 nm) radiation. Mutat Res. 1973; 19:361–364. [PubMed: 4585478]

43. Lee E, Koo K, Berger T. UVB phototherapy and skin cancer risk: a review of the literature. Int J Dermatol. 2005; 44:355–360. [PubMed: 15869531]

44. Hearn RMR, Kerr AC, Rahim KF, Ferguson J, Dawe RS. Incidence of skin cancers in 3867 patients treated with narrow-band ultraviolet B phototherapy. Br J Dermatol. 2008; 159:931–935. [PubMed: 18834483]

45. Osmancevic A, Gillstedt M, Wennberg A-M, Larko O. The risk of skin cancer in psoriasis patients treated with UVB therapy. Acta Derm Venerol. 2013; 94:425–430. [PubMed: 24322826]

46. Tong L, Chuang CC, Wu S, Zuo L. Reactive oxygen species in redox cancer therapy. Cancer Lett. 2015; 367:18–25. [PubMed: 26187782]

47. Verrax J, Calderon PB. Pharmacologic concentrations of ascorbate are achieved by parenteral administration and exhibit antitumoral effects. Free Radic Biol Med. 2009; 47:32–40. [PubMed: 19254759]

48. Köberle B, Masters JRW, Hartley JA, Wood RD. Defective repair of cisplatin-induced DNA damage caused by reduced XPA protein in testicular germ cell tumours. Current Biology. 1999; 9:273–276. [PubMed: 10074455]

49. Laine JP, Mocquet V, Egly JM. TFIIH enzymatic activities in transcription and nucleotide excision repair. Methods Enzymol. 2006; 408:246–263. [PubMed: 16793373]
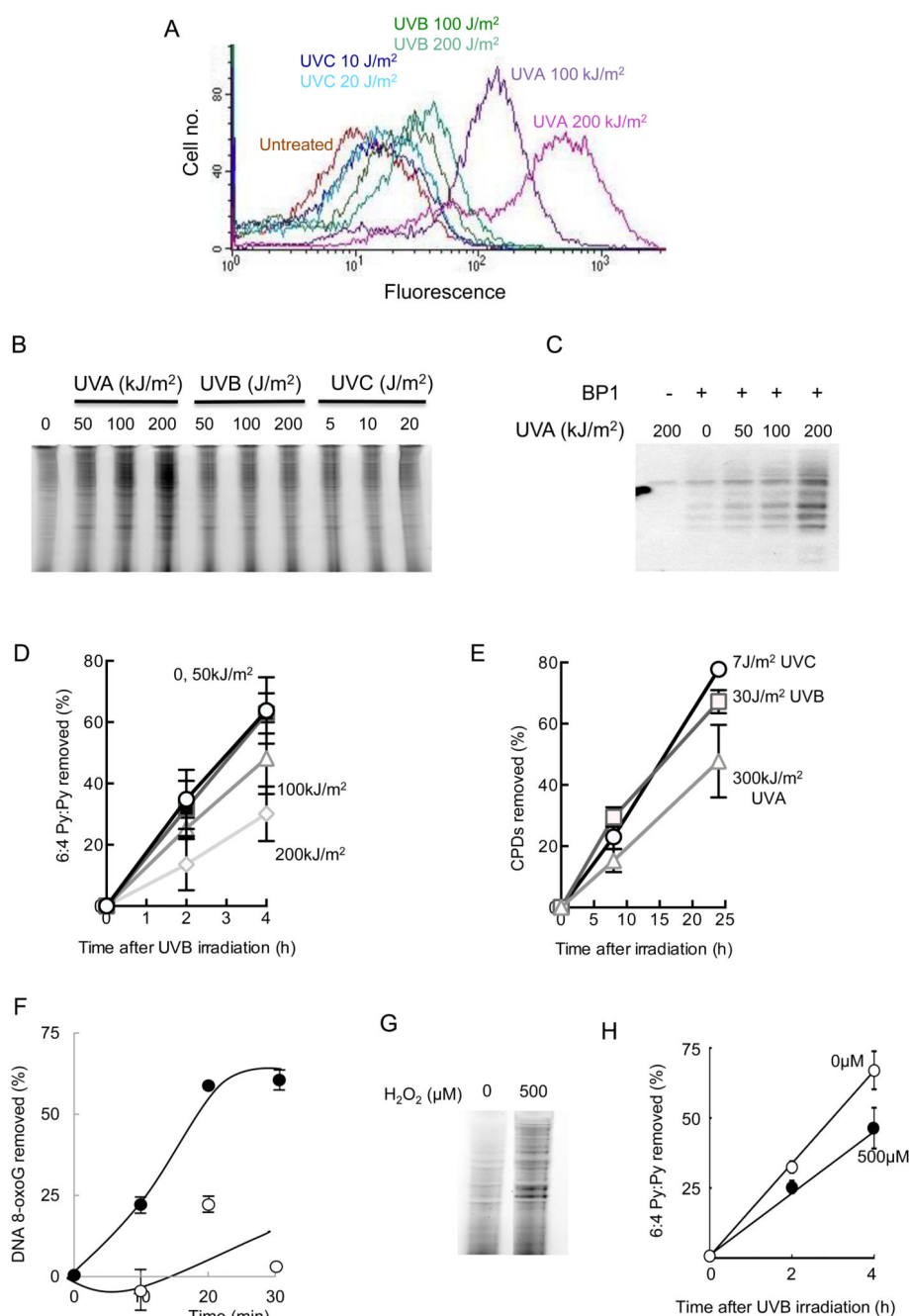
Figure 1. UV-induced ROS, protein oxidation and excision repair

A. Measurement of ROS in HaCaT cells irradiated with UVA, UVB or UVC. ROS were detected using the CM-H$_2$CDFDA probe and FACS analysis.

B. UV-induced protein carbonyls in HaCaT cells. UVA-, UVB- and UVC-irradiated HaCaT cell extracts prepared immediately after irradiation were derivatized with Hydroxylamine Alexa Fluor 647 and separated by PAGE. Carbonylated proteins were visualised at 633 nm.

C. Protein sulfenates in HaCaT cells following UVA irradiation. Proteins in extracts prepared immediately after UVA irradiation were reacted with the biotin-tagged probe 1,3-

cyclopentanedione (BP-1) and captured on streptavidin beads. Eluted proteins were separated by PAGE and transferred to membranes that were probed with streptavidin-HRP.

D. Removal of UVB-induced 6:4 Py:Pys in UVA-irradiated HaCaT cells. Cells were irradiated with 200 J/m^2 UVB and UVA at the doses indicated. DNA was extracted at the times indicated. 6:4 Py:Pys were measured by ELISA. Data for UVB and UVB + 200 kJ/m^2 UVA are means of 9 and 6 determinations, respectively. Other data are the means of at least 2 independent experiments.

E. Removal of CPDs by HaCaT cells. Cells were irradiated with UVA (300 kJ/m^2), UVB (300 J/m^2) or UVC (7 J/m^2) to induce equal numbers of CPDs. DNA was extracted at the times indicated. CPDs were measured by ELISA. The data are the means of 2 independent experiments.

F. DNA 8-oxoG excision by HaCaT cells. Cells were treated with 2.5 mM KBrO$_3$ ± 200 kJ/m^2 UVA. DNA was extracted at the times indicated. DNA 8-oxoG was measured by ELISA. The data are the means of 2 independent experiments.

G. H$_2$O$_2$-induced protein carbonyls in HaCaT cells. Cells were treated with 500 mM H$_2$O$_2$ for 30 min. Extracted proteins were derivatized with Hydroxylamine Alexa Fluor 647 and separated by PAGE. Carbonyls were visualised at 633 nm

H. H$_2$O$_2$-induced inhibition of NER. HaCaT cells treated as in A were irradiated with 200 J/m^2 UVB. DNA was extracted at the times indicated and 6:4 Py:Pys were measured by ELISA.
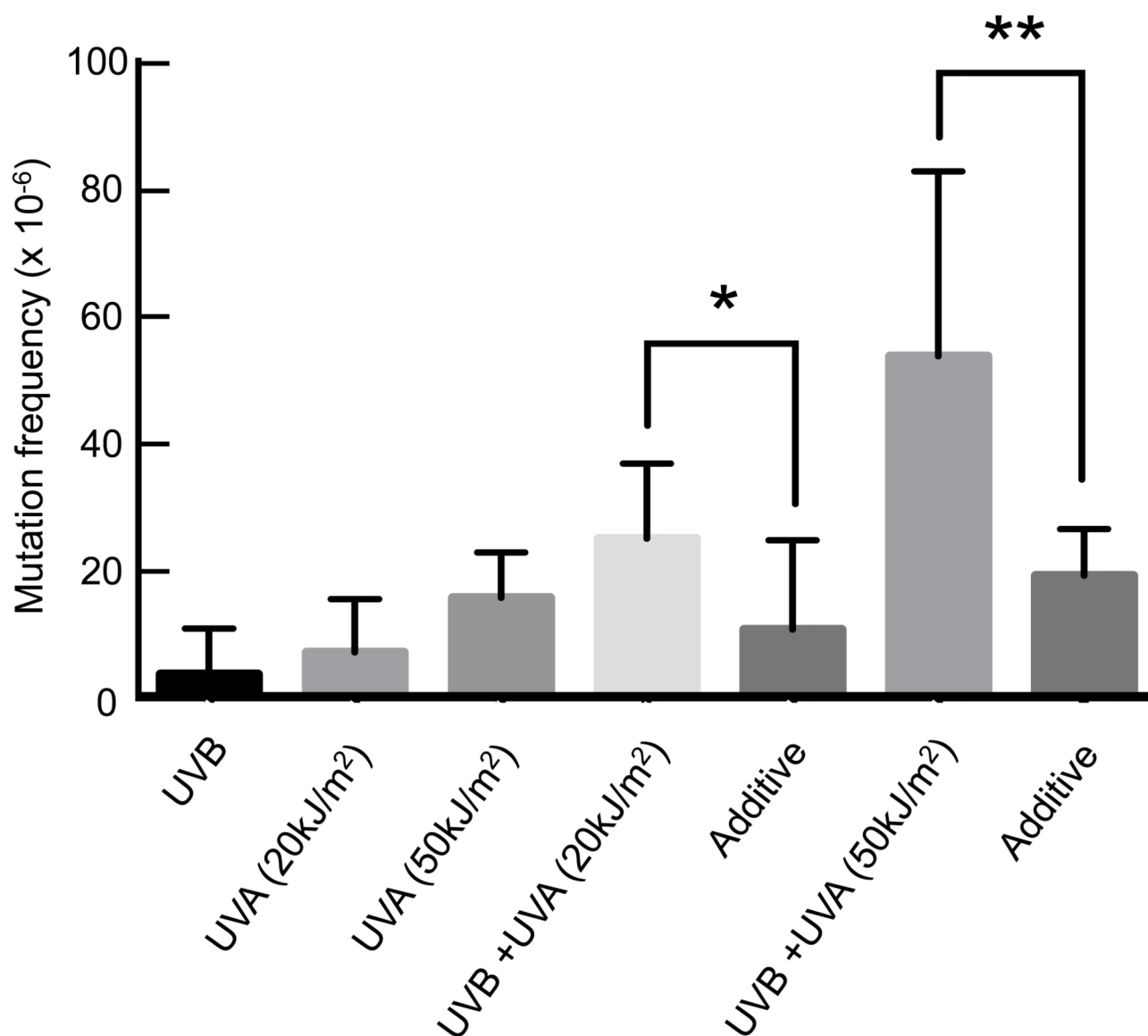
Figure 2. UV-induced mutation

TK gene mutation frequencies in TK6 cells treated with UVB and UVA. F_3TdR mutations induced by 2 J/m^2 UVB, 20 kJ/m^2 UVA and 50 kJ/m^2 UVA are presented along with mutation frequencies in cells treated with UVA/UVB combinations (2 J/m^2 UVB + 20 kJ/m^2 UVA and 2 J/m^2 UVB + 50 kJ/m^2 UVA). 'Additive' values are the aggregate frequency from cells treated with UVA or UVB separately. Comparisons are by a two-tailed Mann-Whitney test. * = p<0.05; ** = p<0.01. A mean background frequency (3.8 ± 1.6) x 10^{-6} has been subtracted from each radiation-induced value.
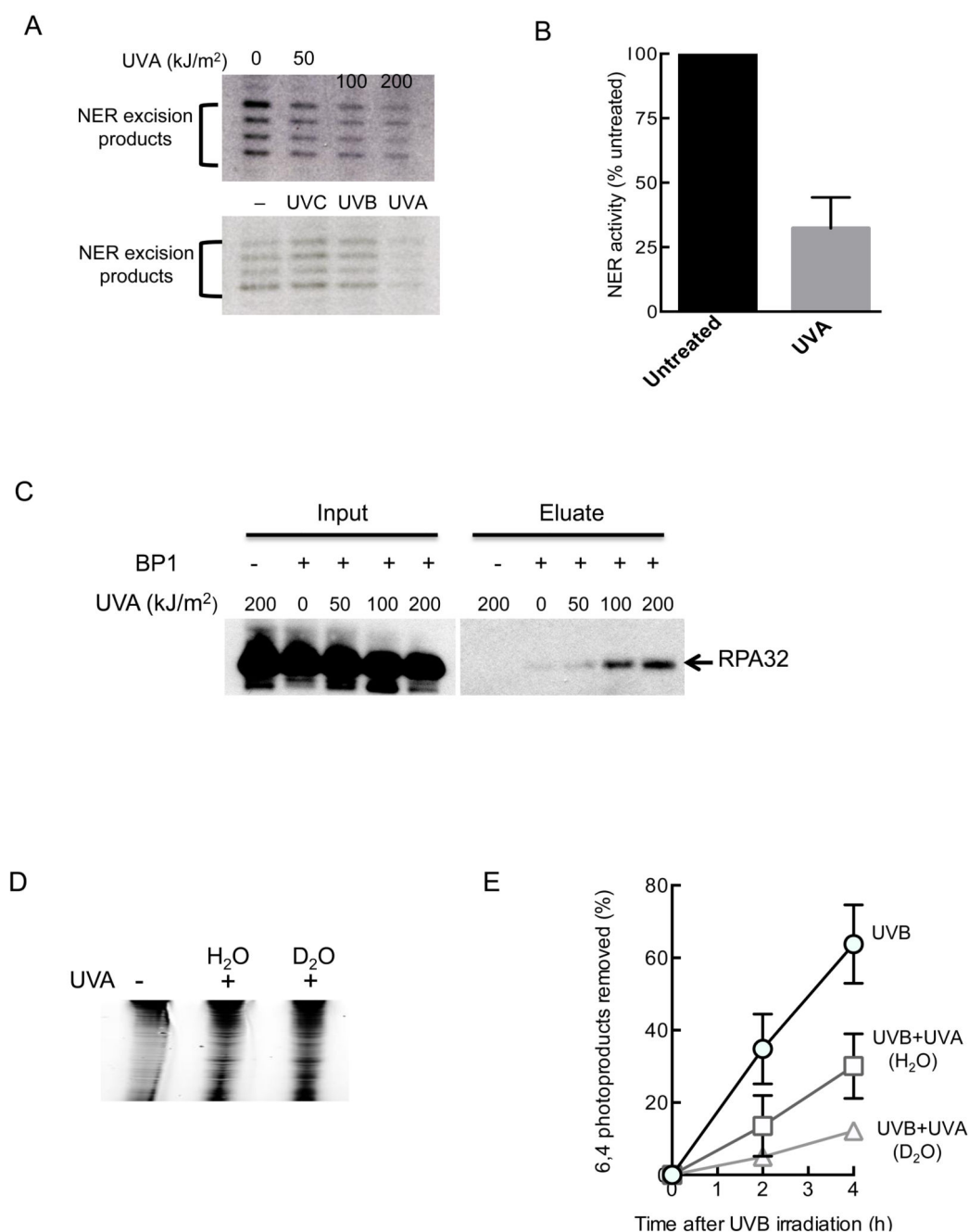
Figure 3. Protein damage & NER *in vitro* and inhibition by $^1O_2$

A. Nuclear extracts prepared from HeLa cells that had been irradiated as indicated were assayed for NER. NER excision products (indicated) were end-radiolabelled and separated by gel electrophoresis.

B. GelDoc quantitation of excision. Means of NER assays with 3 independent extracts from control or UVA (200 kJ/m^2) irradiated HeLa cells.

C. RPA32 sulfenates in UVA-treated HaCaT cells. Following derivatisation with BP-1 and streptavidin bead capture, proteins were recovered, separated by PAGE and immunoblots were probed for RPA32.

Eluate = streptavidin-captured samples; input = samples prior to streptavidin bead loading.

D. $^1O_2$ and UVA-induced protein carbonyls in HaCaT cells. Cells were irradiated with 200 kJ/m^2 UVA in PBS prepared with H_2O or D_2O as indicated. Extracts were prepared and protein carbonyls were derivatized using Hydroxylamine Alexa Fluor 647, separated by PAGE and visualised at 633 nm.

E. $^1O_2$ and NER. HaCaT cells were irradiated with 200 J/m^2 UVB ± 200 kJ/m^2 UVA in PBS prepared with H_2O or D_2O. DNA was extracted at the times indicated and 6:4 Py:Pys measured by ELISA. D_2O data represent the mean of 2 independent experiments.
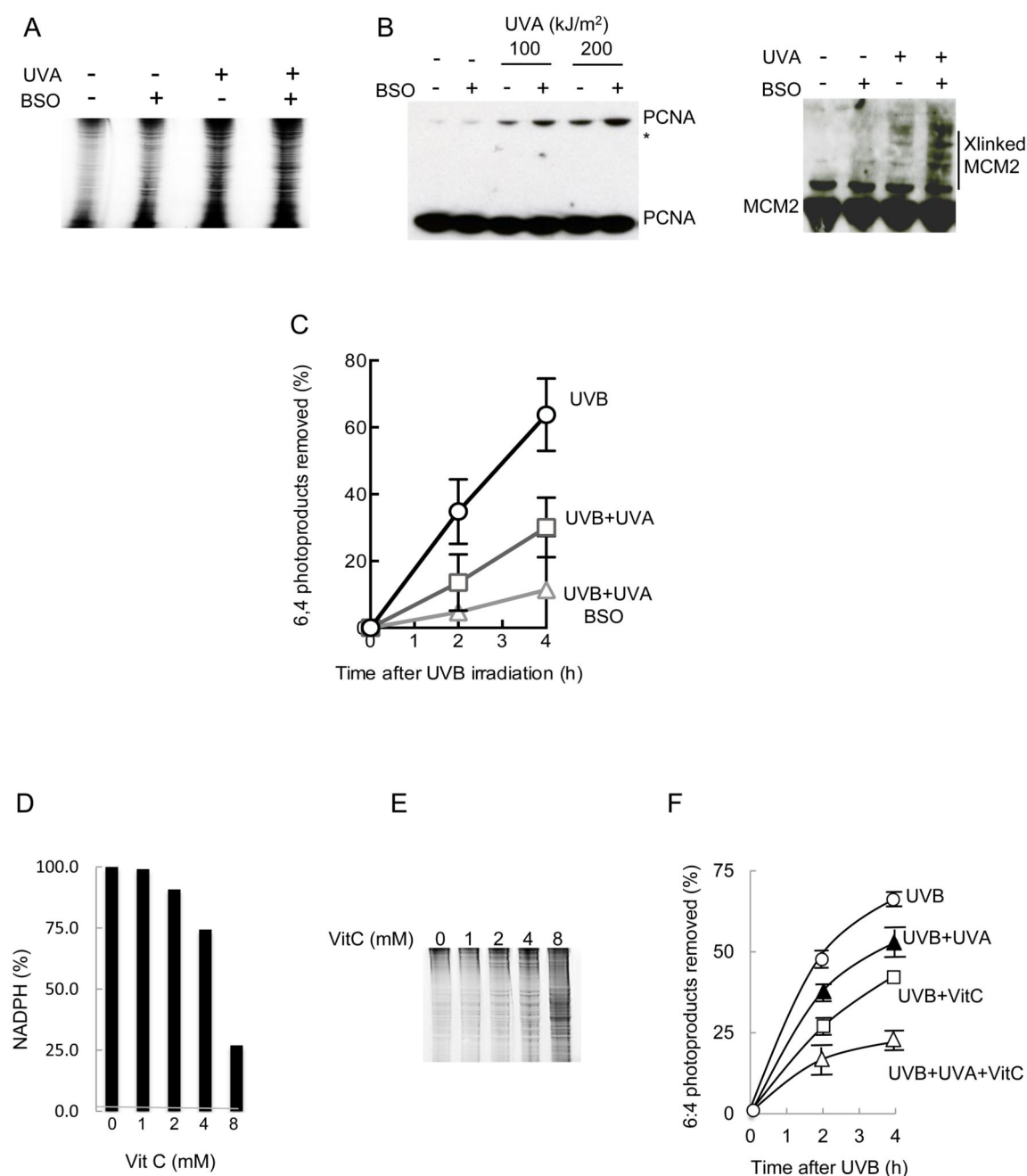
Figure 4. BSO and ascorbate-induced oxidative stress and NER

A. Protein carbonyls in HaCaT cells treated with BSO (2 mM, 24 h) and 200 kJ/m^2 UVA.

B. Covalent PCNA and MCM2 crosslinking. Extracts from HaCaT cells treated with BSO (2 mM, 24 h) and UVA as indicated were analysed by immunoblotting. Crosslinked PCNA (PCNA*) and crosslinked MCM2 species are indicated.

C. NER by BSO-treated cells. ELISA measurements of 6:4 Py:Pys induced by 200 J/m^2 UVB in HaCaT cells pre-treated with 200 kJ/m^2 UVA, or BSO + 200 kJ/m^2 UVA. Data for BSO represent the mean of 2 independent experiments.

D and E. HaCaT cells were incubated in the presence of ascorbate (Vit C) at indicated doses for 3 h.

NADPH levels (d) and protein carbonylation (e) were assayed immediately.

F. Cells were treated with 8 mM ascorbate (Vit C) for 3 hours, irradiated with 150 kJ/m^2 UVA and then incubated in the presence of Vitamin C for the indicated times. Removal of 6:4 photoproducts was measured by ELISA. Data are means of three experiments.
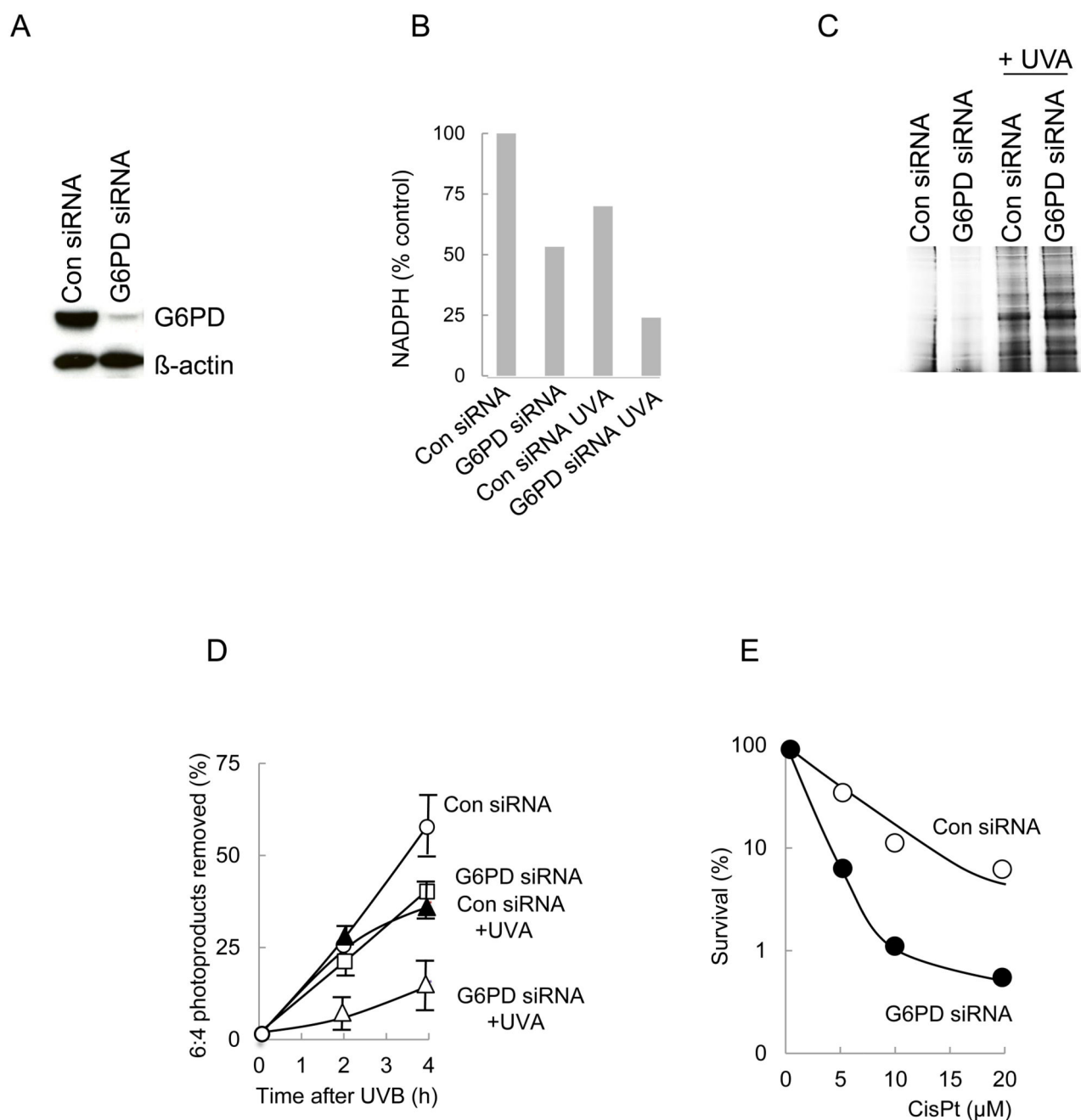
A

B

C

D

E

Figure 5. G6PD silencing and NER

A and B. G6PD immunoblot (a) and NADPH levels (b) in HaCaT cells 72 h after siRNA transfection. NADPH levels were measured 30 min after irradiation with 200 kJ/m^2 UVA.

C. Protein carbonyls in G6PD knockdown HaCaT cells.

D. NER in G6PD knockdown cells measured by ELISA. Cells were irradiated (200 kJ/m^2 UVA) 72 h after transfection. Data are means of three independent experiments.

E. Cisplatin sensitivity. Cells were treated with cisplatin (5h) at indicated doses 72 h after siRNA tranfection. Cells were washed twice with PBS and cell survival was determined by MTT assay 72 h after cisplatin treatment.