17β-Oestradiol attenuates nucleotide excision repair

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Abstract Epidemiological studies strongly suggest associations between chronic exposure to endogenous oestrogens and the development of breast and gynaecological tumours. Two mechanisms by which 17β-oestradiol (E_2) may enhance tumorigenesis are: (i) enhancement of cell proliferation and (ii) the production of reactive, genotoxic metabolites. Here we suggest an additional mechanism, inhibition of DNA repair. The removal of UV-induced thymine dimers from human keratinocytes, reflective of nucleotide excision repair, was significantly attenuated by treatment of cells with E_2. In contrast, treatment with 17α-oestradiol had no effect. Mechanisms are proposed for this effect of E_2, which may contribute to its carcinogenic potential.

Key words: Estradiol; DNA repair; Nucleotide excision repair; Breast cancer; Thymine dimer

1. Introduction

Epidemiological studies have shown that prolonged exposure to oestrogens, particularly 17β-oestradiol (E_2), is one of the primary risk factors for the development of tumours, particularly those of breast and gynaecological tissues [1]. The risk of developing such tumours is related to the length of exposure to endogenous oestrogens, with factors such as early menarche, late first pregnancy, low parity and late menopause all increasing risk [1–3].

Mechanistically, E_2 may exert its carcinogenic actions by at least two principal, and not necessarily mutually exclusive, routes: firstly, through stimulating cell proliferation and secondly via the genotoxicity of metabolites [4]. The growth promoting action of E_2 almost certainly involves positive modulation of cell cycle proteins, at the levels of gene expression and protein function, either through initial interaction with oestrogen receptors (ERs) or possibly via non-genomic mechanisms [5,6]. Hydroxylation of E_2, catalysed by several enzyme activities, particularly cytochrome P450s, can produce the catecholoestrogens, 2- or 4-hydroxyoestradiol [7]. Subsequent redox metabolism of these catechols may ultimately form quinones and reactive oxygen species, which can modify DNA, producing potentially mutagenic lesions, or induce chromosomal aberrations [8,9]. In this report we postulate that E_2 may have an additional property in relation to carcinogenesis, an ability to down-regulate DNA repair. Although not an unknown phenomenon, there are very few reports in the literature relating to an ability of endogenous steroidal sex hormones to modulate DNA repair, certainly an effect of these compounds on nucleotide excision repair (NER) specifically has not been reported [10–12]. In this report we show that treatment of a human keratinocyte cell line with E_2 results in an attenuation of the removal of cyclobutane thymine dimers (T⟨⟩T), as measured by immunoassay. The repair of T⟨⟩T, induced by UV irradiation, occurs exclusively by NER in human cells and therefore provides a convenient method to examine this repair pathway.

2. Materials and methods

2.1. Materials

E_2 and 17α-oestradiol were obtained from Sigma Chemical Co. (Poole, UK). Spontaneously immortalised human keratinocytes, HaCaT cells, were a kind gift from Professor N.E. Fusenig (Deutsches Krebsforschungszentrum, Heidelberg, Germany [13]) and cell culture medium (RPMI 1640) was from Gibco BRL/Life Technologies (Paisley, UK). Foetal bovine serum was stripped of endogenous oestrogens using charcoal as described by Thibodeau et al. [14]. Anti-T⟨⟩T antibody was produced and characterised 'in house' as described by Ahmad et al. [15]. Unless stated otherwise all other materials and chemicals were from Fisher Scientific (Loughborough, UK) or Sigma Chemical Co.

2.2. Exposure of cells to oestradiols and induction of thymine dimers

HaCaT cells were cultured in 6 well plates, at a density of 1×10^4 cells/cm^2 in phenol red-free RPMI 1640 containing 1% charcoal-stripped foetal bovine serum and 2 mM glutamine, overnight at 37°C, 5% CO_2. This medium was then replaced with fresh medium containing various concentrations of oestradiol. After 24 h, the medium was removed and the cells washed with phosphate buffered saline and placed in Hanks' buffered saline solution containing magnesium and calcium salts. To induce T⟨⟩T the cells were then irradiated in a light box with a UV-B source (95% UV-B, 5% UV-A) for a total of 240 mJ/cm^2 (UV-B), after which time the Hanks' buffered saline solution was removed and the cells were returned to 37°C/5% CO_2 in RPMI medium containing 1% charcoal-stripped foetal bovine serum and 2 mM glutamine for defined times.

2.3. Cell viability (MTT assay)

Post UV irradiation, cell viability was measured using the MTT assay, as previously described [16]. The assay is based on the ability of dehydrogenases in viable cells to cleave the tetrazolium ring of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide to yield purple formazan crystals which are insoluble in aqueous solutions. The crystals are subsequently solubilised in dimethyl sulphoxide and the resultant solution is measured spectrophotometrically at 570 nm. An increase or decrease in cell number (and presumably dehydrogenase activity) corresponds to a concomitant change in the amount of formazan crystals formed, giving an indication of the cytotoxicity of the UV-B treatment. In this study, MTT was added to the cells for 2 h

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Abbreviations: E_2, 17β-oestradiol; ER, oestrogen receptor; NER, nucleotide excision repair; T⟨⟩T, cyclobutane thymine dimers

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at a final concentration of 0.5 mg/ml. After the MTT containing medium was removed, the cells were washed with phosphate buffered saline (PBS) and the formazan product dissolved and measured.

2.4. Measurement of thymine dimers in HaCaT genomic DNA

At specified time points, both cells and cell culture medium supernatants were collected. The genomic DNA was extracted from the cells using a Genomic DNA Wizard Preparation kit (Promega UK, Southampton, UK). The protein content of each sample was calculated from the protein fraction of the cells using a BCA protein assay (Perbio Science UK, Tattenhall, UK). Genomic DNA extracted from the samples was analysed for T⟨⟩T content using a direct ELISA protocol based upon that described by Cooke et al. [17]. Briefly, extracted DNA was rendered single stranded by boiling and rapid cooling on ice, then bound to Nunc-Immuno Maxisorp 96 well plates (Life Technologies) coated with poly-L-lysine. Following blocking of the free binding sites, the IgG fraction of a polyclonal rabbit antiserum which detects T⟨⟩T [15] was added at a dilution of 1/1000. Detection was via a peroxidase labelled swine anti-rabbit secondary antibody (Dako, High Wycombe, UK) in conjunction with an orthophenylenediamine substrate solution. The reaction was stopped using 2 M H_2SO_4 and the resultant absorbance was read on a plate reader (Anthos-Labtec 2001, Anthos-Labtec Instruments, East Sussex, UK) at 492 nm. The readings were corrected for background absorbance.

2.5. Measurement of thymine dimers in HaCaT cell culture medium

Quantification of excised T⟨⟩T in the supernatants of irradiated cells was carried out using a competitive ELISA based on that used previously for the urinary analysis of T⟨⟩T [15]. Briefly, a solution of single stranded, UV-C irradiated calf thymus DNA (solid phase antigen) was bound to poly-L-lysine coated 96 well plates. Following blocking of the free binding sites with 4% w/v milk power in PBS, an aliquot of cell culture supernatant was added to the plates, along with the IgG fraction of a polyclonal rabbit antiserum which detects T⟨⟩T, as above, at a final dilution of 1:1000. Primary antibody binding was quantified as described in Section 2.4.

2.6. Immunocytochemical detection of ERα in HaCaT cells

HaCaT cells were grown to confluence in chamber slides with phenol red-free RPMI 1640, plus 2 mM glutamine and 10% v/v stripped serum at 37°C, 5% CO_2. After reaching confluence, medium was removed and replaced with the above medium plus 1% stripped serum overnight. Fresh medium containing 0 (ethanol vehicle only), 200 pM or 5 nM E_2 in ethanol was then added and the cells left for 24 h. After 24 h the culture medium was removed and the cell layer gently washed with PBS, fixed with ethanol (4–5 min) and washed with PBS. Following blocking with serum (2% v/v) for 1 h at 37°C, then washing with PBS, a 1/100 dilution in serum of mouse monoclonal anti-human ERα primary antibody (Dako Cytomation, Ely, UK) was added and incubated for 2 h at 37°C. Following washing with PBS, a 1/40 dilution of FITC conjugated rabbit anti-mouse immunoglobulin secondary antibody (Dako Cytomation) was added and incubated for 0.5 h, then the cells were washed with PBS. The fixed and stained cells were then viewed using fluorescence microscopy.

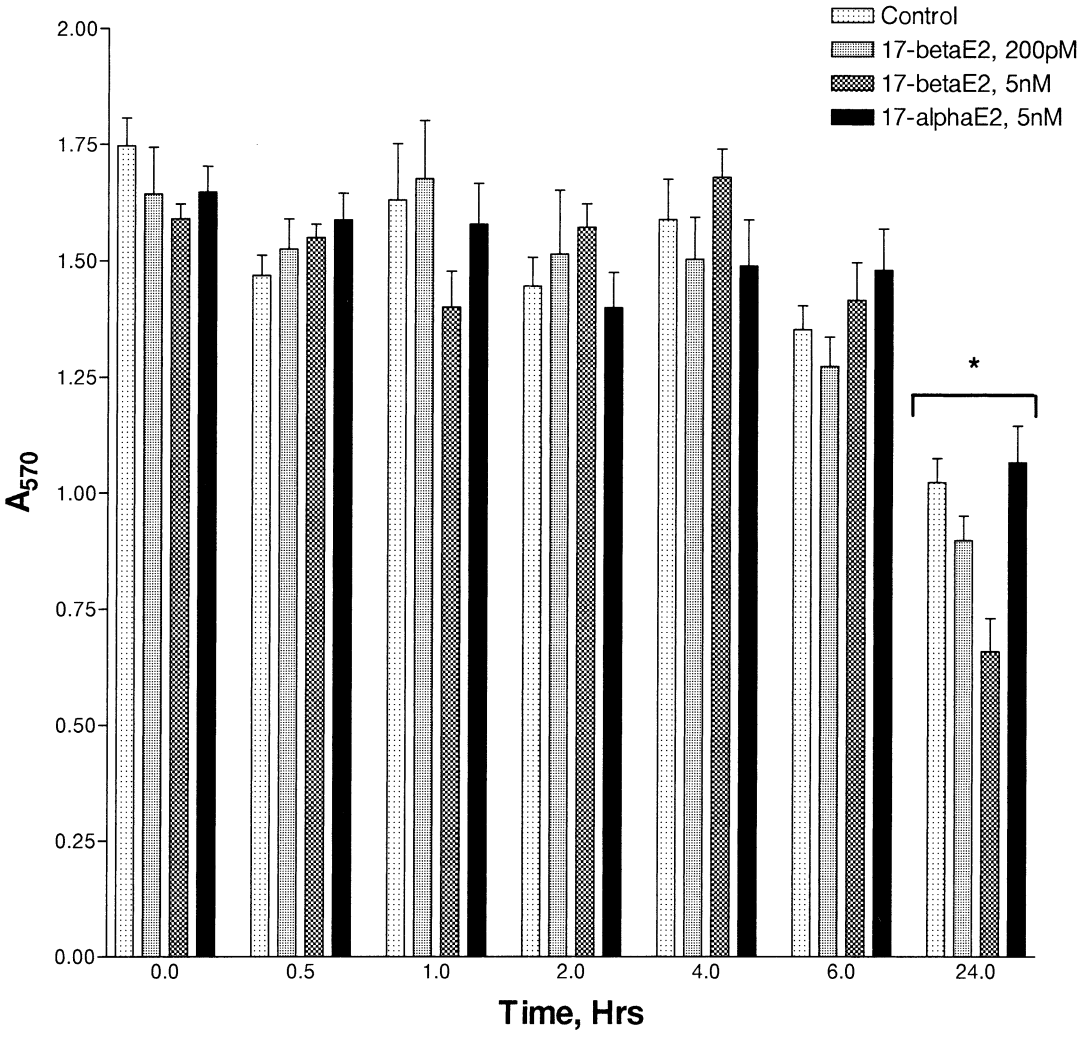

Fig. 1. The effects of E_2 and 17α-oestradiol on the viability of HaCaT cells following UV-B irradiation. The results are presented as mean ± S.D. ($n = 3$); significantly different from 0 h, $^*P \leq 0.05$.

2.7. Statistical analysis

All results were corrected for protein content (mg/ml) and the results are presented as a ratio of non-irradiated samples. In each case the results presented represent the mean of three experiments, with standard deviations presented as a indication of the reproducibility of the assay. Statistical analysis was carried out on each result using Student's *t*-test provided as part of GraphPad Prism software (v2.01; GraphPad Software, San Diego, CA, USA).

3. Results

A significant decrease in viability, as measured by the MTT assay, post UV irradiation, was not detected until 24 h for all treatments compared to the initial time point (Fig. 1; $P < 0.05$). Exposure to E_2 or 17α-oestradiol did not significantly affect post-irradiation viability, irrespective of concentration. The reduction of MTT is predominantly dependent on the activity of cytosolic, pyridine nucleotide dependent dehydrogenases, while some previous observations in the literature have indicated that the MTT assay can be used to report on cell proliferation, with a direct relationship between MTT dye reduction and cell number, thus the assay can be used as a measure of cell viability [18].

The measurement of genomic levels of T⟨⟩T showed an induction of lesions immediately post UV-B exposure and a slow loss of lesions over time, reflective of DNA repair (Fig. 2). As shown, the data are all corrected for protein content to account for cell number and also levels of lesion are shown relative to a control not exposed to UV radiation or E_2. On this basis, immediately following UV irradiation, levels of T⟨⟩T at least doubled in irradiated cells irrespective of E_2 treatment (Fig. 2). By 24 h, the level of T⟨⟩T returned almost to control, unirradiated levels. Since T⟨⟩T are removed by NER, this removal reflects the activity of this pathway. Although some loss of cell viability may have an impact on the data at the 24 h time point, since genomic DNA is extracted from the adherent cell population, and non-viable cells will detach, the 24 h data should represent lesion levels in viable cells. Pre-treatment of cells with both 200 pM (physio-

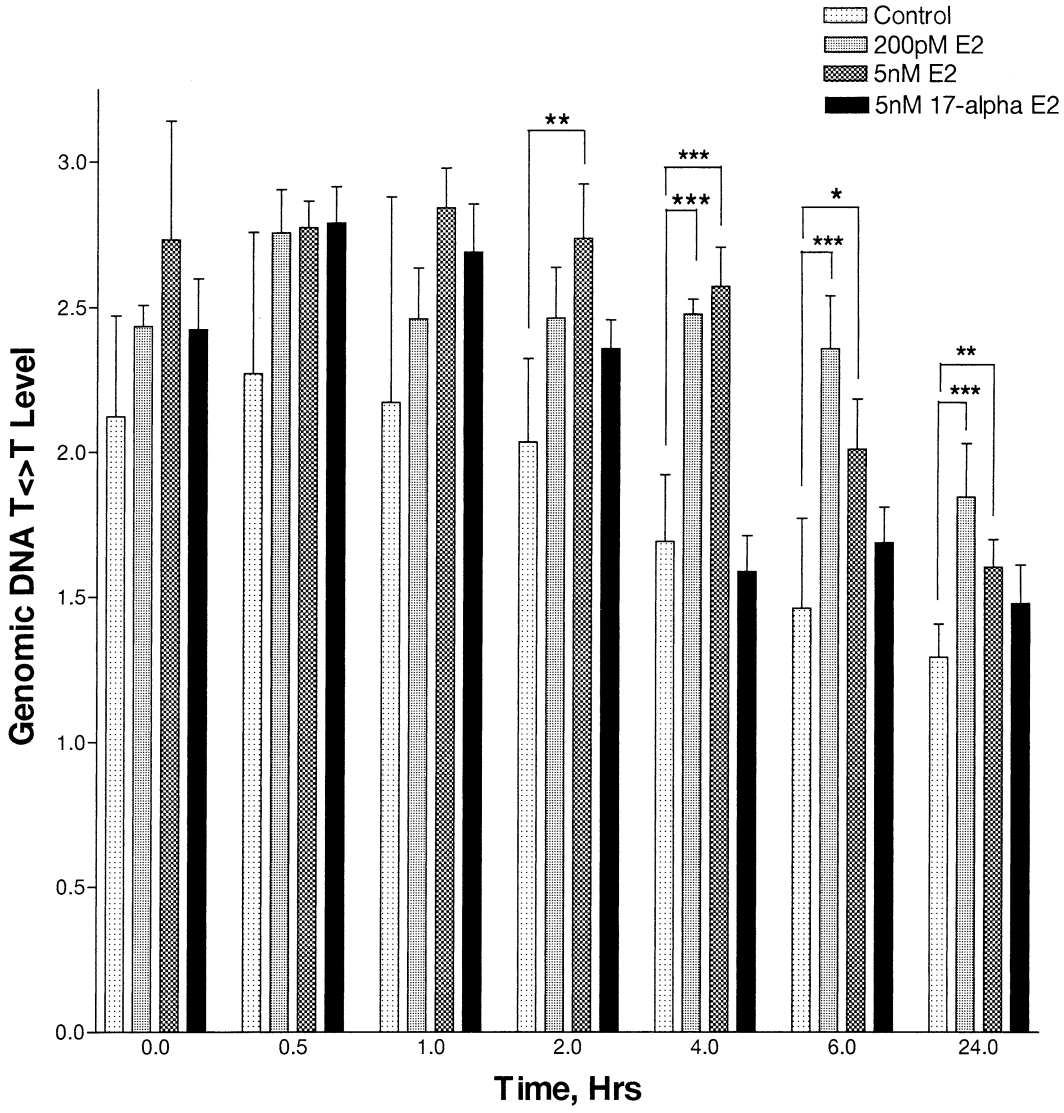

Fig. 2. The effects of exposure of HaCaT cells to E_2 and 17α-oestradiol on thymine dimer content in genomic DNA following UV-B irradiation. The results are expressed as a fold increase in thymine dimer content over non-irradiated samples and the results are normalised for protein content. Results are presented as mean ± S.D. ($n = 3$ experiments); significantly different compared to control, $*P \leq 0.05$, $**P \leq 0.02$, $***P \leq 0.01$.

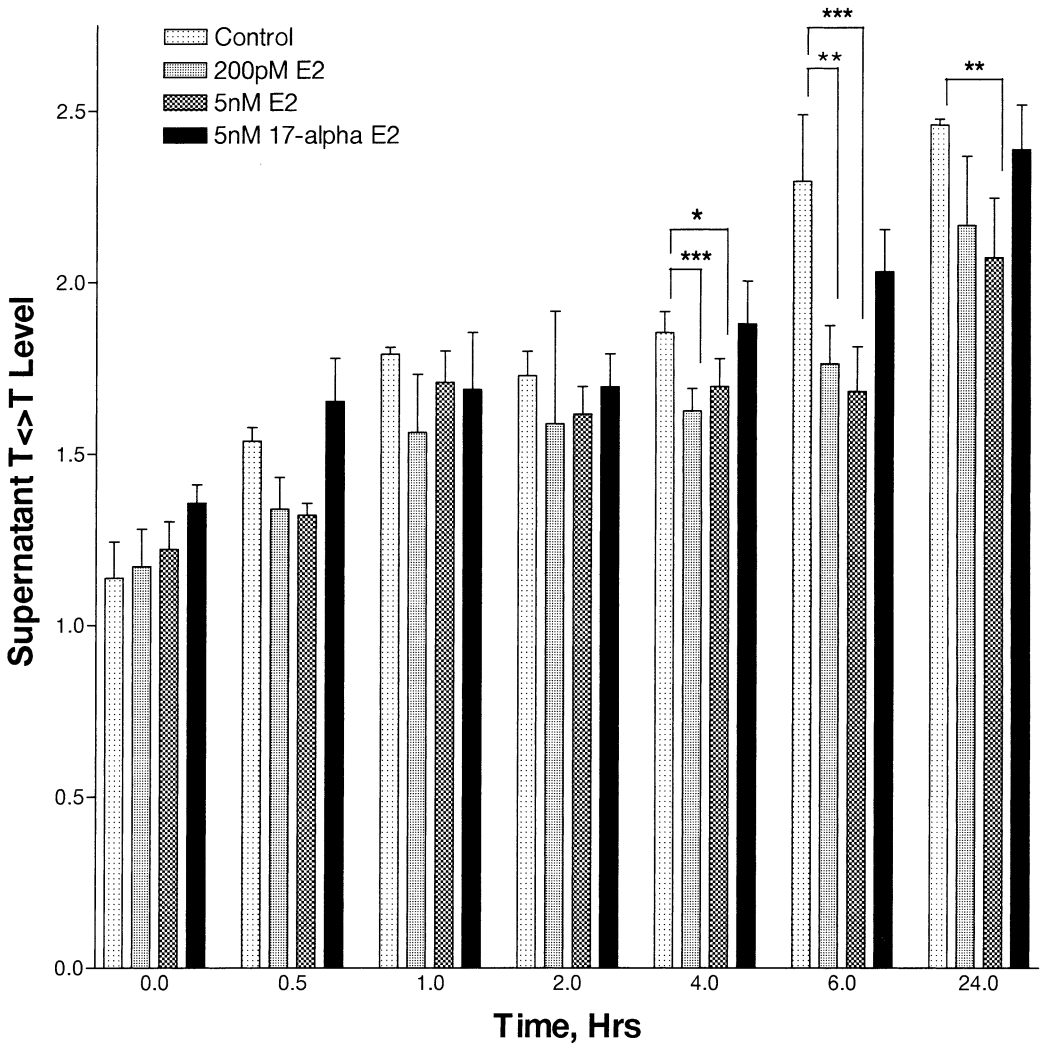

Fig. 3. The effects of E_2 and 17α-oestradiol on the release of thymine dimers into the medium of HaCaT cells exposed to UV-B irradiation. Experimental details are described in the legend to Fig. 2. Results are presented as mean ± S.D. ($n = 3$ experiments); significantly different compared to control, $*P \leq 0.05$, $**P \leq 0.02$, $***P \leq 0.01$.

logical level) and 5 nM (supra-physiological level) E_2 induced an appreciable delay in the removal of T⟨⟩T, which became significantly different at 2 h. In contrast, 17α-oestradiol, a physiologically inactive isomer of E_2, did not delay removal of T⟨⟩T and removal paralleled that of the non-E_2 treated cells (Fig. 2). The removal of T⟨⟩T from genomic DNA was reflected by supernatant levels of excreted repair products, T⟨⟩T, analysed by competitive ELISA (Fig. 3). In this case, E_2 pre-treatment suppressed excretion of T⟨⟩T compared to control cells and as with genomic DNA T⟨⟩T levels, 17α-oestradiol pre-treatment paralleled the excretion pattern of untreated cells. A significant difference between E_2 pre-treated and untreated cells only became significant at 4 h and later time points; at 24 h, loss of cell viability may begin to impact on the supernatant levels of lesions. In all of these experiments, oestrogen exposure was performed prior to irradiating the cells, and cells were not incubated in a medium containing supplemental oestrogens after irradiation.

A qualitative immunocytochemical analysis revealed that HaCaT cells grown under the experimental conditions expressed ERα. A suitable quantitative analysis of expression was not feasible, in part because culturing HaCaT cells with E_2 led to notable detachment of the cells from the chamber slides; modulation of cellular adherence by E_2 treatment has been noted in the literature [19]. However, a purely visual examination suggested that those cells that had been exposed to exogenous E_2 displayed more intense fluorescence.

4. Discussion

Collectively, the data strongly suggest that E_2 attenuates the repair of T⟨⟩T in HaCaT cells and, by implication, attenuates the activity of NER. The inhibition of DNA repair by endogenous steroidal sex hormones, including E_2 and progesterone, was reported in the early 1970s, but since then few reports of this phenomenon have been noted [10,11]. However, these early studies used hormone concentrations that were well above those found physiologically, unlike in the present study where we used concentrations within (200 pM) or relatively close to (5 nM) physiological levels in healthy females. A few other studies, in vivo or using synthetic oestrogen, provide further, albeit limited, evidence for the potential of female sex steroids to modulate DNA repair, but do not specifically refer to NER [12,20].

Collectively, several observations from the literature imply that E_2 has the potential to attenuate NER and hence support our findings in this study. Functional inactivation of p53 through relocalisation to the cytoplasm has been observed in E_2 treated MCF-7 cells [21]. This would seem to be significant as p53 is important for efficient global genome repair of T⟨⟩T by NER, through modulation of activity/expression of repair proteins or induction of cell cycle arrest [22,23]. Evidently, if p53 function is impaired in some way, cell cycle checkpoint function may also be impaired. By acting as a proliferating agent, E_2 may drive cell proliferation to diminish cell cycle checkpoint function and accompanying DNA repair activities, which has been postulated to contribute to the induction of genetic errors in the genesis of breast and endometrial cancers [1]. Furthermore, E_2 can up-regulate *mdm2* expression; MDM2 binds to activated p53 and targets it for transport to the cytoplasm and degradation by the proteasome [24]. The targeted degradation of repair proteins by the proteasome is an additional mechanism by which E_2 may modulate NER, apart from any uncharacterised effect it may have on NER repair protein expression. Recent data suggest that E_2 increases the expression of components of the proteasome complex [25]; the ubiquitin–proteasome pathway for protein degradation is a route whereby NER may be regulated [26]. Elevated expression of the anti-apoptotic protein Bcl-2 is also reported to increase proteasome activity [27]. At a concentration of 1 nM, E_2 induces expression of Bcl-2 in the ER positive cell line MCF-7 [28]. Cells over-expressing Bcl-2 show an attenuation of NER and repair of oxidative DNA lesions such as thymine glycol and 8-oxo-2′-deoxyguanosine [29,30]. This suggests a further connection between E_2 exposure and the down-regulation of DNA repair, although the mechanistic links are presently unclear. It is also possible that the anti-apoptotic action of Bcl-2 may be important in promoting the survival of cells harbouring unrepaired DNA, thus providing an additional carcinogenic mode of action for E_2.

While the above discussion is speculative, what was evident mechanistically from the present study was that 17α-oestradiol apparently had no effect on the repair of T⟨⟩T, implying that E_2 may attenuate NER through interaction with ER, either classical nuclear receptors or ectopically expressed ER [31,32]. Immunocytochemistry revealed that HaCaT cells can and do express ERα, consistent with observations of the expression of functional ER in dermal keratinocytes suggested by several studies [33–35]. Whether this DNA repair modulating effect of E_2 is limited to a few cell types, perhaps only those harbouring functional ER, remains to be examined.

We suggest that an inhibitory action of E_2 on NER and perhaps other DNA repair pathways may have pathological significance and implies an additional mechanism whereby this compound may influence carcinogenesis in responsive tissues. There are several reports of the positive influence of impaired DNA repair on the process of carcinogenesis to imply that this concept is feasible [36,37] and although the data are sparse, a relationship between gender (possibly related to hormonal influence), carcinogenesis and the repair of UV induced DNA damage has been reported [38]. However, there is no doubt that a considerable amount of work remains to be done with regard to exactly how E_2 may modulate NER and whether it has similar effects on other DNA repair pathways. Furthermore, the effects of related steroidal hormones may also warrant investigation and hopefully this report will provide fresh impetus for such work.

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