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EFFECTS OF THE SEX HORMONES AND THEIR MODULATORS ON DNA DAMAGE IN SKIN CELLS MEASURED IN THE COMET ASSAY

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Abstract. Aims. Hormones can react through receptors and/or possibly through the production of oxygen radicals. The Comet assay examined DNA damage produced by the androgens, testosterone (TES) and dihydrotestosterone (DHT), and the oestrogens, diethylstilbestrol (DES) and β-oestradiol in $NCTC^{2544}$ cells (keratinocyte cell line) and primary keratinocytes. **Materials and methods.** The TES and DHT responses were evaluated with and without the androgen modulator, cyproterone acetate (CPA), and DES and β-oestradiol with and without the oestrogen modulator, tamoxifen (TAM). Oxygen radical-type effects in keratinocytes were evaluated with hydrogen peroxide (H_20_2) by itself and in combination with catalase (CAT), CPA and TAM. **Results and discussion.** TES, DHT and DES produced positive responses, and the extent of the DNA damage produced was reduced by the appropriate modulator. The DNA damage induced by H_20_2 was reduced by CAT, CPA, and TAM. **Conclusions.** These observations suggest by analogy, that oxygen radicals are involved in DNA damage produced by the hormones.

Key words: androgens, oestrogens, cyproterone acetate, tamoxifen, hydrogen peroxide, skin cells, oxygen radical damage

Rezumat. Obiective. Hormonii pot acționa prin intermedierea receptorilor şi/sau prin producerea de radicali de oxigen. Testul „Comet" examinează alterarea DNA produsă de androgeni, testosteron (TES) şi dihidrotestosteron (DHT) şi de estrogeni, dietilstilbestrol (DES) şi β-estradiol în cazul celulelor $NTCT^{2544}$ (linii celulare de keratinocite) şi al keratinocitelor primare. **Material şi metode.** Răspunsul TES şi DHT a fost evaluat cu şi fără androgen modulator, ciproteron acetat (CPA), iar DES şi β-estradiol cu şi fără estrogen modulator, tamoxifen (TAM). Efectele radicalilor de oxigen asupra keratinocitelor au fost evaluate cu peroxid de hidrogen (H_2O_2) singur sau în combinație cu catalaza (CAT), CPA şi TAM. **Rezultate şi discuții.** TES, DHT si DES au determinat un răspuns pozitiv, iar extinderea alterărilor cauzate DNA au fost reduse de către modulatorul corespunzător. Alterarea DNA indusă de H_2O_2 a fost redusă de către CAT, CPA şi TAM. **Concluzii.** Aceste observații sugerează prin analogie că radicalii de oxigen sunt implicați în alterarea DNA produsă de către hormoni.

Cuvinte cheie: androgeni, estrogeni, ciproteron acetat, tamoxifen, peroxid de hidrogen, celulele pielii, alterări produse de către radicalii de oxigen

INTRODUCTION

Skin is a heterogeneous tissue that has multiple functions including protection against infection and ultraviolet light, temperature regulation, the control of water balance, and excretion. Another important function of skin is that it acts as an endocrine organ. Although it has been recognized from receptor distribution and related actions that steroid hormones may have significant effects on many elements of skin

physiology, it is still unclear exactly how they exert their actions (1, 2).

Steroid hormones can act in various ways. The classical mode of action is the activation of specific intracellular receptors (3). In the absence of ligand, steroid receptors are associated with an inhibitory heat shock protein (HSP) (4). When the ligand binds to the receptor, a conformational change occurs, resulting in the dissociation of the HSP and the formation of stable dimers (5). These ligand-activated receptor dimers tightly associate with specific consensus DNA sequences or hormone response elements (HREs). The steroid receptor complex then interacts with other cellular components to either activate or suppress transcription of the target gene in a promoter- and cell-specific manner (6).

The sex steroids, the androgens and oestrogens, have important roles in the skin of both males and females. The roles of androgens on the hair follicle and sebaceous gland have been well characterized (7). Although their role in skin maintenance is less clear, the levels of androgen receptors are much higher in genital skin, and the expression of aromatase in the skin appears to be modulated by androgens (8, 9).

Aromatase is required for the conversion of androgens to estrogens, the terminal ligands in steroid biosynthesis, by the loss of the C-19 methyl group and the formation of an aromatic ring. However, the vascular nature of the skin would also enable metabolism of hormones through lymphocyte P450 metabolism, as it is known that lymphocytes are metabolically competent (10).

For many years it has been recognized that oestrogens are important in the maintenance of human skin (1). Oestrogens have been shown to increase mitotic activity in the epidermis of both rodents and man (11,12). Autoradiographical studies with injected tritiated estradiol have demonstrated that this hormone is localized in the epidermis of the skin of mice, suggesting that keratinocytes are targets for estrogen action (13).

Other studies have demonstrated that keratinocytes express an estrogen receptor (ER), and oestrogens have been implicated in the progression of basal cell and squamous cell carcinomas (14,15,16). Also oestrogens have been implicated in prostate and breast cancer (17,18)

There are two separate and distinct intracellular ERs, ERα and ERβ, which bind 17β-estradiol with a similar affinity (19). More recently it has been shown that in some tissues oestrogens may signal via a membrane receptor (20). Thornton et al. have demonstrated that human keratinocytes predominately express ERβ *in situ*, suggesting that oestrogen effects on epidermal keratinocytes are mediated via ERβ (21). Until recently it was thought there was only one oestrogen receptor, i.e., ERα.

However, oestrogens can also act through oxygen radical mechanisms. Cytochromes P450-catalysed metabolism of the aromatic phenolic group of oestrogens to ortho-quinones occurs by two single-electron transfer reactions generating two superoxide anion radicals from two oxygen molecules (22). Disproportionation of

the two superoxide anions to H_20_2 and oxygen by the superoxide dismutases lowers the localized level of oxidative stress, although quinones and semi-quinones can also damage DNA directly (23, 24). The Comet assay has been used to demonstrate that various oestrogenic compounds, at doses somewhat higher than those which activate oestrogen receptors, can give rise to DNA damage in human sperm and lymphocytes that is mediated through oxygen radical damage, and that this damage can be abolished or diminished by the endogenous antioxidant catalase (CAT) (25). Also work has suggested that there may be an epigenetic transgenerational action of endocrine disruptors on male fertility, also at doses higher than those which activate oestrogen receptors (26). Hiraku *et al* showed that catechol oestrogens induced formation of 8-oxo-7,8-dihydro-2'-deoxyguanosine in MCF-7 breast cells in culture highlighting their potential to initiate carcinogenesis through oxidative DNA damage (27). What still is not known is whether various androgenic compounds can also give rise to DNA damage, and, if produced, whether such damage can be abolished or diminished by steroid modulators/ receptor antagonists. In previous studies it has also been shown that antioxidants and vitamins can modulate responses to hormones (10,25,28). It follows that at the higher doses at which the steroids can produce oxygen radical damage, then receptor antagonist/hormone modulators could diminish effects.

In this study, we have evaluated the induction of DNA damage in skin by estrogens and androgens and the possible involvement of oxygen radicals in generating the damage. We have examined the DNA damage produced by H_2O_2, two androgens, testosterone (TES) and dihydrotestoserone (DHT), and two oestrogens, diethylstilbestrol (DES) and β-oestradiol, in $NCTC^{2544}$ cells (keratinocyte cell line) and primary keratinocytes. As modulators of the responses, we have not only examined CAT, but also cyproterone acetate (CPA) and tamoxifen (TAM) as exogenous agents associated with androgens and oestrogens, respectively. CPA is a widely used anti-androgenic (i.e. reduces response to androgens) drug used to treat prostate cancer, which may be induced by oxidative stress, while TAM is an anti-oestrogenic (i.e. reduces response to oestrogens) drug widely used against breast cancer and is known to protect membranes, including nuclear membranes, against free radical intermediates and products of lipid peroxidation (29,30). Severe oxidative stress and DNA damage requires the production of millimolar concentrations of H_2O_2 in cells (31). DNA damage from H_2O_2 is most likely caused by the localized production of highly-reactive hydroxyl radicals by both the Fenton and Haber-Weiss reactions ($Fe^{+2} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\bullet}$; $O_2 + H_2O_2 \rightarrow O_2 + OH^- + OH^{\bullet}$) (32).

MATERIALS AND METHODS

Cell culture

Chemicals

All chemicals used were of cell culture grade and supplied by Sigma–Aldridge (Gillingham, Dorset, UK), unless otherwise stated. Biowest supplied Dulbecco's modified Eagles' medium (DMEM) and

fetal calf serum (FCS), while Keratinocyte Serum Free (KSF) medium and supplements, amphotericin B, L-glutamine, penicillin/ streptomycin, and trypsin/EDTA (ethylenediamine tetraacetic acid) were obtained from Gibco (Paisely, Scotland, UK).

Growth media

DMEM was supplemented with 2 mmol/ml L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B and 10% FCS. KSF medium was supplemented with bovine growth factor, epidermal growth factor, 100 IU/ml penicillin and 100 μg/ml streptomycin.

NCTC^{2544} cells

NCTC^{2544} cells were cultured in DMEM supplemented with 10% FCS until confluent. Confluent cultures were subsequently sub-cultured at a ratio of 1:5. These cells are epithelial-like skin keratinocytes. They show a good expression of cytochrome P450-dependent enzymatic activities and are thus a useful model for cytotoxicological studies. The depositor of this cell line was Prof. M Ferro, University of Genoa, Italy.

Skin samples

Human skin samples were obtained from routine plastic surgery. Samples were collected no more that 6 hr post-surgery and were transported to the laboratory in transport medium and processed immediately.

Isolation of primary epidermal keratinocytes

The skin samples were washed thoroughly with sterile phosphate-buffered saline (PBS) and the excess dermis was removed. The epidermis and a small amount of dermis were incubated in dispase (2.4 U/ml) (Roche, Lewes, UK) for 18 hr at 4°C. The epidermis was then peeled off from the remaining dermis using forceps and washed in PBS containing 100 IU/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml amphotericin B. The epidermis was transferred into a 5 ml universal tube containing 600 μl – 1 ml trypsin/ EDTA and incubated at 37°C for 5 min before briefly vortexing. The trypsin/EDTA was transferred into 10 ml KSF medium containing 10% FCS. Fresh trypsin/ EDTA was added to the epidermis and incubated, as described above for a further 2 – 3 times. The cell suspension was centrifuged at 13,000 rpm for 10 min in a swinging bucket centrifuge (MSE, Newport Pagnall, Bucks.). The supernatant was removed, 1 ml of fresh medium without serum was added, and the pellet was re-suspended. The cells were seeded in an appropriate culture dish, normally a 25 cm^2 or 75 cm^2 flask, depending on the number of cells. The medium was changed every 2-3 days until the cells became approximately 70% confluent at 1-2 weeks. Thereafter, the cells were sub-cultured at a ratio of 1:2.

Cell preparation for Comet assay

Both NCTC^{2544} and primary epidermal keratinocytes were trypsinsed, 5 ml DMEM supplemented with 10% FCS or KSF media, respectively, added, and cells centrifuged at 1400 rpm (850 g) for 15 min in a swinging-bucket centrifuge. The cell pellet was re-suspended in 5 ml DMEM or KSF medium.

Cell treatment and DNA damage analysis

Test chemicals

All chemicals were obtained from Sigma Aldrich, Gillingham, Dorset

unless otherwise stated. Testosterone (TES; CAS: 58-22-0], dihydrotestosterone (DHT; CAS No:521-18-6) cyproterone acetate (CPA; CAS: 427-51-0) tamoxifen (TAM ; CAS: 10540-29-1), hydrogen peroxide (H_2O_2; CAS: 9001-05-2) and catalase (CAT; CAS: 9001-05-2) were obtained from Sigma, while diethylstilbestrol (DES; CAS: 56-53-1) and β-estradiol (CAS: 50-28-2) were obtained from Aldrich (Gillingham, Dorset, UK). All steroid chemicals were dissolved in dimethyl sulphoxide (DMSO) CAS: 67-68-5.

Treatment of cells

The cells were treated (approximately 2 $x10^5$ cells /ml)with the compounds for 30 min, except in the case of H_2O_2, in which case the treatment was for 15 min. Doses for DES, β-oestradiol, TES, DHT, TAM, CPA and H_2O_2 were determined in $NCTC^{2544}$ from a preliminary single dose ranging study shown in Table 1 (see Results section). Two independent studies were carried out with the keratinocyte cell line to evaluate the reproducibility of data.

For the assays conducted with primary keratinocytes, a single study was performed with DES, β-oestradiol and TES, along with the appropriate modulator, in order to determine if the response patterns for primary cells were similar to those observed with the cell line. There was no further access to other samples.

Table 1. Preliminary study to determine dose response relationships after treatment of $NCTC^{2544}$ cell line with hormones and their modulators.

TES

	Control	10μM/ml	50μM/ml	100μM/ml	150μM/ml
Mean	2.17	12.40	22.40	21.42	24.13
SD	6.66	19.71	28.40	24.91	29.64
SE	0.94	2.79	4.02	3.52	4.19
Median	0.66	1.0	6.62	11.82	10.56

DHT

	Control	10μM/ml	50 μM/ml	100μM/ml	150μl/ml
Mean	2.31	7.92	11.56	23.62	24.16
SD	7.87	18.44	16.19	27.17	34.91
SE	1.11	2.61	2.29	3.84	4.94
Median	0.81	1.43	8.79	16.94	4.55

CPA

	Control	10μM/ml	50μM/ml	75μM/ml	100μM/ml	150μM/ml
Mean	1.62	15.70	15.45	23.27	24.91	15.27
SD	2.36	17.40	15.89	21.11	21.97	17.03
SE	0.33	2.47	2.25	2.99	3.11	2.41
Median	0.82	10.50	12.19	20.71	19.63	8.03

DES

	Conrtol	10μM/ml	50μM/ml	75μM/ml	100μM/ml	150μM/ml
Mean	1.59	6.36	17.69	26.81	28.37	23.07
SD	1.82	13.67	24.94	28.42	31.32	25.40
SE	0.26	1.93	3.53	4.02	4.43	3.59
Median	0.74	1.17	3.88	13.07	11.85	6.27

β-estradiol

	Control	10μM/ml	50μM/ml	75μM/ml	100μM/ml	150μM/ml
Mean	2.62	5.97	8.59	17.12	26.97	11.55
SD	2.49	14.85	14.3	24.42	25.62	21.22
SE	0.35	2.1	2.03	3.45	3.62	3.00
Median	2.14	0.6	1.62	3.125	23.98	1.11

TAM

	Control	10μM/ml	50μM/ml	75μM/ml	100μM/ml	150μM/ml
Mean	1.96	6.15	12.95	23.70	28.00	28.2
SD	3.11	10.98	14.49	17.90	20.50	17.8
SE	0.44	1.55	2.05	2.54	2.90	2.52
Median	1.11	0.67	6.41	2120	18.99	22.80

H_2O_2

	Control	10μM/ml	20μM/ml	30μM/ml	40μM/ml	50μM/ml
Mean	0.72	0.71	6.26	21.49	34.41	50.45
SD	1.08	0.55	3.31	10.86	14.79	22.32
SE	0.15	0.08	0.47	1.54	2.09	3.16
Median	0.38	0.41	4.35	19.19	33.99	50.29

Comet assay

The majority of the chemicals for the Comet assay were supplied by BRL Life Technologies (Gillingham, Dorset, UK). Fluka Chemicals (Gillingham, Dorset, UK) supplied sodium chloride and sodium hydroxide, and Tris and ethidium bromide were obtained from Sigma (Poole, Dorset, UK).

The cells were tested for viability before the start and after the completion of the treatment using the trypan blue dye exclusion assay (33, 34). The preparation of slides for the Comet assay and the subsequent electrophoresis and staining were carried out as previously described for lymphocytes, using slight modifications of the methods described by Singh *et al* and Tice et al. (25,35,36,37).

The slides were scored using an image analysis system (Kinetic Imaging, Liverpool, UK) attached to a fluorescence microscope (Leica, Wetzlar, Germany) equipped with appropriate filters. The microscope was connected to a computer through a charge coupled device (CCD) camera to

transport images to appropriate software (Komet 4.0; Kinetic Imaging) for analysis. The final magnification was 200x (20x objective plus10x ocular). The parameter used for evaluating DNA damage was tail moment (arbitrary units). The image analysis software automatically generated the tail moment, which is the tail length multiplied by the staining intensity. Fifty cells were analyzed per slide, i.e. 100 cells at each concentration per study.

Statistical Analysis

Statistical analysis was performed on median tail moment values for each experimental sample, and the mean ± S.E. results are shown. The tail moment data for the keratinocytes violated the normality and equal variance test required for parametric analysis. Thus, pair-wise comparisons of the treatment groups versus the control were conducted using the Mann-Whitney test.

RESULTS

The viability of the all treated cells exceeded 85%, so excluding artifactual responses in the Comet assay due to the toxic effect of the chemicals.

The doses of, TES, DHT, CPA DES, β-oestradiol TAM, and H_2O_2 used to treat the cells were determined from a preliminary dose ranging study shown in Table 1. All compounds produced positive responses. Doses used in the main study were selected from the exponential part of the dose response-relationship.

The results of the main study are summarized in Table 2.

The androgens, DES and H_2O_2 caused DNA damage in the keratinocyte cell line and primary keratinocytes as measured by increases in the comet tail moments. Androgen treatments conducted in combination with CPA in the cell line reduced the extent of DNA damage. Low concentrations (50 μM/ml) of TAM increased the DNA damage caused by the estrogens, while the extent of this increase was reduced at higher TAM concentrations (100μ M/ml).

In addition, higher concentrations of CAT, TAM and CPA all reduced the DNA damage produced by H_2O_2.

Figures 1 and 2 show typical results that were obtained with the NCTC^{2544} keratinocyte cell line.

Results for the individual experiments are shown in the Tables 3-12.

Elevated levels of DNA damage were produced when the cells were treated with the androgens. TES treatment resulted in an increase in the Comet tail moments, which were reduced when treatments were conducted in combination with CPA (table 3).

The greatest increases in tail moments were produced in exposures conducted with DHT, but the overall response was similar to that with TES, and like TES, the extent of damage was reduced by CPA (table 4).

Table 2. Summary of responses in the Comet Assay testing the effects of sex hormones, their modulators, H_2O_2 and catalase on DNA damage in human primary keratinocytes and the keratinocyte cell line NCTC [2544]

Compounds	No modulator		Tamoxifen (TAM) (micromoles/ml)						Cyproterone acetate (CPA) (micromoles/ml)						Catalase (CAT) (IU/ml)					
			50		75		100		50		75		100		50		100		150	
	Pk	Cl	Pk	Cl	Pk	Cl	Pk	Cl	Pk	Cl	Pk	Cl	Pk	Cl	Pk	Cl	Pk	Cl	Pk	Cl
Testosterone (TES) (100 micromoles)	ns	+ +							+	ns -	+	- -	ns	ns -						
Dihydroxytestosterone (DHT) (100 micromoles/ml)		+ +								- -		- -		- -						
Diethyl stilboestrol(DES) (100 micromoles/ml)	+	+ +	+	+ +	+	+ +	+	ns -												
β-oestradiol (100 micromoles/ml)	ns	ns ns	+	+ +	+	+ +	+	ns ns												
Hydrogen peroxide(H_2O_2) (30 micromoles/ml)		+ + + + + +		ns +		- ns		- -		+ns		- ns		- -		- -		- -		- -

\+ Significant elevation; for treatment conducted with hormone/ H_2O_2 alone, relative to control; for co-treatments, relative to hormone/ H_2O_2 alone.

\- For co-treatments, reduction relative to response with hormone/ H_2O_2 alone

ns Non-significant differences; comparisons as defined above

+ , -, ns Data from one study

++, etc Data from more than one study

Pk: Primary keratinocytes Cl : Keratinocyte cell line NCTC [2544]

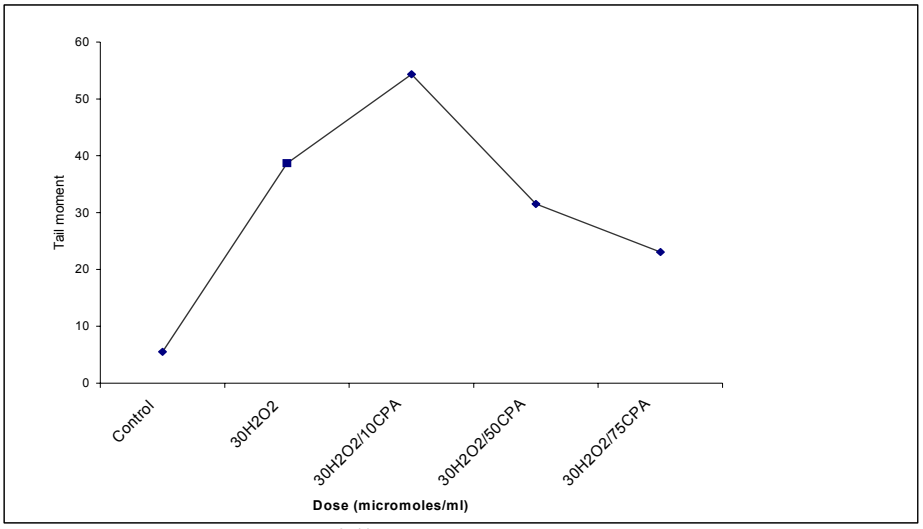

Fig. 1. The response of NCTC^{2544} cells to H_20_2 and its modification with CPA. Doses given in micromoles/ml

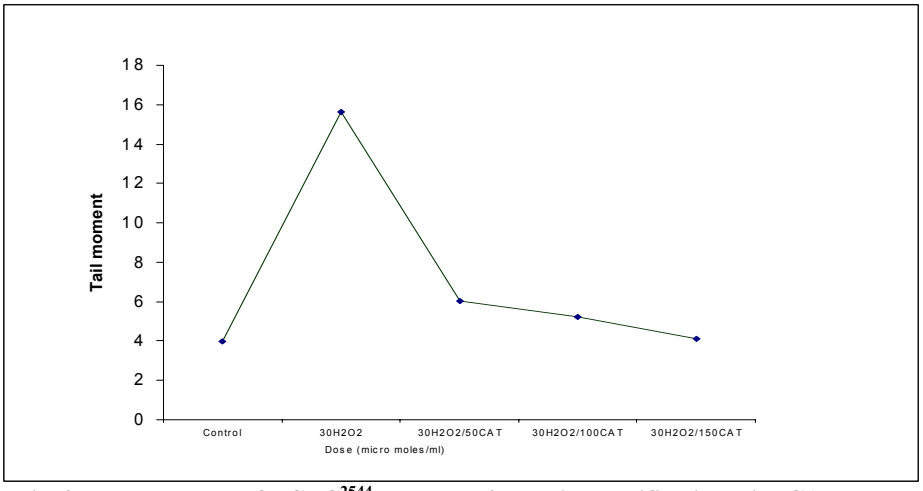

Fig. 2 . The response of NCTC^{2544} cells to H_20_2 and its modification with CAT. Doses given in micromoles/ml

Table 3.Response of NCTC [2544] keratinocyte cells to TES and CPA

Study 1					
Doses	a	b	c	d	e
	Control	100 TES	100 TES + 50 CPA	100 TES + 75 CPA	100 TES + 100 CPA
Mean	1.44	18.38	18.25	6.40	4.88
SD	3.09	29.57	28.11	15.53	8.41
SE	0.31	2.96	2.81	1.55	0.84
Median	0.53	1.86	3.44	1.23	1.90
		b-a	b-c	b-d	b-e
		p<0.001	NS	p<0.05	NS

Study 2				
a	b	c	d	e
Control	100 TES	100 TES + 50 CPA	100 TES + 75 CPA	100 TES + 100 CPA
5.60	16.09	8.61	9.46	5.68
11.6	21.91	18.88	19.89	11.75
1.16	2.19	1.88	1.98	1.17
0.93	4.70	1.00	0.98	1.02
	b-a	b-c	b-d	b-e
	p<0.001	p<0.001	p<0.001	p<.001

All concentrations in micromoles/ml

DES induced an increase in DNA damage in the keratinocyte cell line, the extent of which was increased in treatments conducted in combination with the lower doses of TAM (table 5). 100 μM/ml TAM reduced the extent of DNA damage caused by 100 μM DES. Treatment with 100 μM/ml β-estradiol only marginally increased DNA damage in one study and was positive in the other; however, the effect of co-treatment with TAM was similar to that seen with DES (table 6). The effect of H_2O_2 on comet tail moments and its modification by CPA and TAM are shown in Tables 7 and 8 respectively. Higher doses of both the modulators diminished the extent of DNA damage produced by H_2O_2. The DNA damage produced by H_2O_2 was also decreased by co-treatment with CAT (table 9).

Studies with primary keratinocytes

TES only marginally increased DNA damage above control values but CPA increased the DNA damage produced by TES in primary keratinocytes; however, as was seen in the cell line, higher doses of CPA tended to decrease the extent of the damage (table 10). Co-treatments conducted with the two highest doses of CPA produced levels of DNA damage that were returning towards control values

Table 4. Response of NCTC [2544] keratinocyte cells to DHT and CPA

Study 1					
Doses	a	b	c	d	e
	Control	100 DHT	100 DHT+50 CPA	100 DHT+75 CPA	100 DHT+ 75 CPA
Mean	3.13	20.22	7.93	6.03	2.94
SD	8.32	25.53	13.53	13.25	6.86
SE	0.83	2.55	1.35	1.37	0.68
Median	0.53	5.15	1.80	0.98	0.66
		b-a	b-c	b-d	b-e
		p<0.001	p<0.01	p<0.001	p<0.001

Study 2				
a	b	c	d	e
Control	100 DHT	100 DHT + 50 CPA	100 DHT + 75 CPA	100 DHT + 75 CPA
0.98	17.32	7.88	14.10	3.91
1.29	31.80	18.74	30.72	13.54
0.12	3.18	1.87	3.07	1.35
0.44	1.64	0.75	0.70	0.55
	b-a	b-c	b-d	b-e
	p<0.001	p<0.05	p<0.05	p<0.05

All concentrations in micromoles/ml

Table 5. Response of NCTC[2544] keratinocyte cells to DES and TAM

Study 1					
	a	b	c	d	E
Doses	Control	100 DES	100DES+ 50 TAM	100DES+ 75 TAM	100DES+ 100 TAM
Mean	1.08	10.26	23.25	13.84	14.14
SD	1.04	18.18	24.49	17.00	19.84
SE	0.10	1.82	2.45	1.70	1.98
Median	0.71	2.52	13.55	6.14	4.89
		b-a	b-c	b-d	b-e
		p<0.001	p<0.001	p<0.001	NS

Study 2				
a	b	c	d	e
Control	100 DES	100DES+ 50 TAM	100DES+ 75 TAM	100DES+ 100 TAM
4.52	7.05	24.74	19.01	4.08
5.44	8.35	25.69	18.06	4.66
0.54	0.83	2.57	1.81	0.46
2.59	4.88	17.15	15.72	2.49
	b-a	b-c	b-d	b-e
	p<0.01	p<0.001	p<0.001	p<0.01

All concentrations in micromoles/ml

Table 6. Response of NCTC [2544] keratinocyte cells to β oestradiol and TAM

Study 1					
	a	b	c	d	e
Doses	Control	100 β- oestradiol	β-oestradiol + 50 TAM	β-oestradiol + 75 TAM	β-oestradiol + 100 TAM
Mean	3.37	3.71	8.36	10.05	5.19
SD	2.68	5.94	12.99	11.66	8.85
SE	0.27	0.59	1.30	1.17	0.88
Median	2.82	2.96	4.00	6.67	3.34
		b-a	b-c	b-d	b-e
		NS	p<0.001	p<0.001	NS

Study 2				
a	b	c	d	e
Control	100 β-oestradiol	β-oestradiol + 50 TAM	β-oestradiol + 75 TAM	β-oestradiol + 100 TAM
1.78	13.18	29.05	23.72	9.87
2.53	22.77	32.38	25.00	13.36
0.25	2.28	3.24	2.50	1.34
1.27	3.05	17.23	13.51	3.98
	b-a	b-c	b-d	b-e
	NS	P<0.001	p<0.001	NS

All concentrations in micromoles/ml

Table 7. Response of NCTC [2544] keratinocyte cells to H_2O_2 and CPA

Study 1					
	a	b	c	d	e
Doses	Control	30 H_2O_2	30 H_2O_2+50 CPA	30 H_2O_2+75 CPA	30 H_2O_2+100 CPA
Mean	5.51	38.68	54.34	31.49	23.05
SD	2.97	25.41	22.15	24.23	17.37
SE	0.30	2.54	2.21	2.42	1.74
Median	5	36.59	25.84	25.84	19.06
		b-a	b-c	b-d	b-e
		P<0.001	P<0.001	P<0.05	P<0.001

Study 2				
a	b	c	d	e
Control	30 H_2O_2	30 H_2O_2 +50 CPA	30 H_2O_2 +75 CPA	30 H_2O_2 +100 CPA
3.43	28.50	32.43	29.06	25.52
2.34	14.30	19.50	19.10	17.58
0.23	1.43	19.10	1.91	1.76
3.14	25.4	31.33	28.33	20.45
	b-a	b-c	b-d	b-e
	p<0.001	NS	NS	p<0.001

All concentrations in micromoles/ml

Table 8. Response of NCTC [2544] keratinocyte cells to H_2O_2 and TAM

Study 1					
	a	b	c	d	e
Doses	Control	30 H_2O_2	30 H_2O_2+50 TAM	30 H_2O_2+75 TAM	30 H_2O_2+ 100 TAM
Mean	3.91	33.89	39.07	26.36	20.46
SD	3.39	23.11	21.66	19.32	13.063
SE	0.34	2.31	2.17	1.93	1.31
Median	3.55	31.62	43.93	19.90	17.29
		b-a	b-c	b-d	b-e
		P<0.001	NS	P<0.05	P<0.001

Study 2				
a	b	c	d	e
Study 1	30 H_2O_2	30 H_2O_2 +50 TAM	30 H_2O_2 +75 TAM	30 H_2O_2 + 100 TAM
3.65	16.81	32.21	17.38	4.96
5.34	18.02	22.61	18.22	6.94
0.54	1.80	2.26	1.82	0.69
2.38	7.93	1.82	8.34	3.07
	b-a	b-c	b-d	b-e
	P<0.001	P<0.001	NS	P<0.001

All concentrations in micromoles

Table 9. Response of NCTC [2544] keratinocyte cells to H_2O_2 and CAT

Study 1					
Doses	a	b	c	d	e
	Control	30H_2O_2	30 H_2O_2+50 CAT	30 H_2O_2+100 CAT	30 H_2O_2+150 CAT
Mean	1.27	7.61	2.29	1.69	2.02
SD	1.39	8.85	2.31	1.36	1.87
SE	0.14	0.88	0.23	0.14	0.19
Median	0.96	4.68	1.67	1.34	1.61
		b-a	b-c	b-d	b-e
		p<.001	p<.001	p<.001	p<.001

Study 2				
a	b	c	d	e
Control	30 H_2O_2	30 H_2O_2+50 CAT	30 H_2O_2+100 CAT	30 H_2O_2+150 CAT
3.98	15.64	6.02	5.24	4.13
3.96	20.64	9.44	3.60	5.14
0.40	2.06	0.94	0.36	0.51
3.16	5.13	3.03	4.77	3.59
	b-a	b-c	b-d	b-e
	p<.001	p<.05	p<.001	p<.001

Concentration of H_2O_2 in micromoles/ml and CAT in IU/ml

Table 10. Response of primary keratinocyte to TES and CPA

Study 1					
	a	b	c	d	e
Doses	Control	100 TES	100 TES+50 CPA	100 TES +75 CPA	100 TES + 100 CPA
Mean	1.73	2.40	8.20	5.09	4.18
SD	2.59	4.22	15.64	6.21	6.24
SE	0.37	0.59	2.21	0.87	0.88
Median	0.52	0.76	3.04	2.48	1.10
		b-a	b-c	b-d	b-e
		NS	P<.01	p<.01	NS

All concentrations in micromoles/ml

Data were not available for DHT and CPA due to the limited supply of samples. Table 11 and 12 show the results of experiments conducted with DES and β-estradiol. Tail moments with DES alone were significantly different from control values, and co-treatments conducted with TAM significantly increased these values; however, as seen with the cell line, higher doses of TAM reduced the DNA damage. Treatment with β-oestradiol alone did not alter the tail moments significantly from control values. In combination with TAM, all values were significantly greater than with β-oestradiol alone, but were dose-responsively reduced with increasing TAM doses.

DISCUSSION

Aerobic organisms are under constant threat from the harmful effects of reactive oxygen species and oxidative stress results when pro-oxidant effects exceed antioxidant effects (38,39). Hormones have been linked to the production of reactive oxygen species during their metabolism (40). The activity of cytochromes P450 results in the oxidation of the aromatic phenolic group of estrogens to ortho-quinones causing the generation of two anionic superoxide radicals (22). Similarly high levels of androgens such as TES have been linked to the production of free radicals (29). Moreover, these hormones are metabolically linked to each other since TES can be converted to β-oestradiol, the reaction being catalysed by the enzyme, aromatase. The E_2 ER of epithelial tissue is capable of producing oxidative DNA damage through an ER-mediated mechanism and these effects were reduced by the anti-oestrogen, TAM (41). These authors also concluded that androgens are capable of increasing oxidative stress through increased mitochondrial activity and altering the level of enzymes involved in maintenance of the pro-oxidant/ antioxidant balance.

Table 11. Response of primary keratinocyte cells to DES and TAM

Study 1					
	a	b	c	d	e
Doses	Control	100 micromol/ ml DES	100 micromol/ml DES + 50 micromol/ ml TAM	100 micromol/ml DES + 75 micromol/ ml TAM	100micromol/ml DES + 100 micromol/ml TAM
Mean	4.96	6.06	20.38	15.45	10.28
SD	5.72	9.50	22.48	14.49	11.96
SE	0.80	1.34	3.18	2.04	1.69
Median	2.09	2.7	14.04	12.35	5.92
		b-a	b-c	b-d	b-e
		p<.01	p<.01	p<.001	p<.001

All concentrations in micromoles/ml

Table 12. Response of primary keratinocyte cells to β-estradiol and TAM

Study 1					
	a	b	c	d	e
Doses	Control	100micromol/ ml β-estradiol	100 β-estradiol + 50micromol/ml Tam	100micromol/ml β- estradiol + 75micromol/ml Tam	100micromol/ml β-estradiol + 100micromol/ml Tam
Mean	3.83	4.98	19.03	17.65	11.58
SD	5.87	9.44	15.95	15.45	9.84
SE	0.83	1.37	2.25	2.19	1.39
Median	0.61	1.99	15.32	13.75	8.66
		b-a	b-c	b-d	b-e
		NS	p<.001	p<.001	p<.001

All concentrations in micromoles/ml

In the preliminary dose ranging studies with the NCTC[2544] keratinocyte cell line, all compounds examined produced DNA damage on their own, even the modulators. Cyproterone acetate has been suggested to be a genotoxic carcinogen (42) and TAM to operate through a genotoxic mechanism (43). Anderson *et al.* (10) have shown catalase to be positive in the Comet assay. Hormonal steroids in general are known to be genotoxic (25). Doses for DES, β-oestradiol, H_2O_2 and catalase were in the same range as those used in a previous study with human lymphocytes and sperm (25).

The present study used the keratinocyte cell line NCTC[2544], a cell line possessing a relatively high level of antioxidant capacity (44), yet, the androgens and DES were able to increase DNA damage in the primary keratinocytes, as well as in the keratinocyte cell line, NCTC[2544] although not with statistical significance for TES and β-oestradiol. β-oestradiol also

produced only marginal but non-significant increases in DNA damage in the cell line. Although the primary keratinocyte data do not exactly mirror the NCTC[2544] cell line data, the qualitative pattern of response is similar i.e. when co-treatments were conducted in both cell types with modulators of hormonal activity, low doses of the modulators tended to increase the levels of DNA damage; however, higher doses resulted in a reduction towards control values. It is realized that repetition of data with primary keratinocytes would be useful, but trends in response are the same in both cell types.

It is surprising that combining two genotoxins in this way can reduce responses at higher doses, with exacerbation at lower doses, but it has been shown on previous occasions with other modulators, such as flavonoids on food mutagens in human blood and sperm in the Comet assay and antioxidants in sperm and blood with oestrogens (25, 28). This is possibly because effects are additive or synergistic at low doses and antagonistic at high doses and may correspond to the pro-oxidant/ antioxidant status at different concentrations as shown for vitamin C (10).

Similar responses were obtained in NCTC[2544] cells with H_2O_2 and CAT and with H_2O_2 and the androgen and oestrogen modulators. By analogy, these observations suggest that some of the DNA damage produced by the androgens and estrogens in skin cells may be due to the generation of oxygen free radicals.

CONCLUSIONS

TES, DHT and DES produced positive responses, and the extent of the DNA damage produced was reduced by the appropriate modulator. The DNA damage induced by H_20_2 was reduced by CAT, CPA, and TAM. These observations suggest by analogy, that oxygen radicals are involved in DNA damage produced by the hormones.

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