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MITOGEN-INDUCED TOPOISOMERASE II SYNTHESIS PRECEDES DNA SYNTHESIS IN HUMAN BREAST CANCER CELLS

Richard J. Epstein* and Paul J. Smith

Department of Clinical Oncology & Radiotherapeutics
MRC Centre, Hills Rd, Cambridge CB2 2QH, U.K.

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Cells released from quiescence exhibit increased levels of the DNA-modifying enzyme topoisomerase II, a nuclear protein which is also a target for antitumour drugs such as VP-16 (etoposide) and m-AMSA (4',9'-acridinylamino-methanesulfon-m-anisidide). By using Western blotting, DNA-protein crosslinking and drug-induced DNA cleavage to detect topoisomerase II, we show here that oestrogen stimulation of T-47D human breast cancer cells results in increased cellular enzyme content at least 4hr prior to enhancement of DNA synthesis. Taken in conjunction with previous findings, these results suggest that oestrogen enhances topoisomerase II synthesis within a G_1-phase cell subset.

In addition to its recognised roles in chromatin structure (1) and mitosis (2), topoisomerase II has been implicated in DNA replication (3) and gene transcription (4). Proliferating cells exhibit greater topoisomerase II activity than quiescent cells (5), but it is not clear whether this distinction reflects predominant enhancement of replicative or transcriptional activity. Furthermore, it is also unclear to what extent this enhancement depends upon new enzyme synthesis or enzyme activation, both of which have been implicated as important regulatory mechanisms in different cell systems.

We have previously shown that VP-16-induced DNA cleavage may be used as a measure of topoisomerase II availability in intact viable T-47D cells (10) and that such cleavage is antagonised by the protein synthesis inhibitor cycloheximide but not by the DNA α-polymerase inhibitor aphidicolin (11). These findings raised the possibility that the enhancement of topoisomerase-II-induced DNA cleavage seen

*Address for correspondence: R.J. Epstein, Department of Microbiology & Molecular Genetics, Harvard Medical School, Dana-Farber Cancer Institute, 44 Binney St, Boston,MA 02115.

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with oestrogen stimulation (10) indicated an early response of topoisomerase II to mitogenic activation. We now present further evidence confirming that oestrogen-stimulated T-47D cells synthesise topoisomerase II prior to enhancement of DNA synthesis.

MATERIALS AND METHODS

T-47D cell culture and hormone stimulation were carried out as previously described (10). Measurement of drug-induced DNA cleavage was performed using a modified version (11) of the alkaline unwinding technique described by Kanter and Schwartz (12). DNA-protein crosslinking induced by **m**-AMSA was used to measure extractable topoisomerase II; this was quantified using the filter-binding technique of Minford *et al* (13), with the modification that linear end-labelled pBR322 plasmid DNA (a gift of Dr P. Rabbitts) was used as substrate. To prepare crude protein extracts, cells were grown in 175cm^2 flasks and harvested using 0.02% EDTA after oestrogen/ethanol exposure. The permeabilised nuclear pellets were extracted for 20 mins at 4^{o}C with buffer containing 350mM NaCl and then centrifuged. The clarified supernatant was added to reaction volumes (200µl) containing 15ng radiolabelled DNA and **m**-AMSA (20µM) and incubated at 37^{o}C for 25 mins. DNA-protein crosslinking (giving rise to filter retention) was determined as described previously (13).

Western blotting was performed by separating proteins on a discontinuous gel using the method of Laemmli (14). Nuclear extracts were prepared as described above. The gel to be blotted was put on a 0.45µm pore size Millipore nitrocellulose sheet and the assembly placed in a Transphor chamber. Transfer of resolved proteins from gels to nitrocellulose filter paper was as described by Towbin (15). Protein transfer was performed for 4hr at 4^{o}C at a constant current of 0.5A using a solution containing 12.5mM Tris, 0.2M glycine (pH 8.5) and 20% methanol (w/v) as the electrode buffer. After transfer, additional protein binding sites on the nitrocellulose were blocked by incubation of the paper for 16hr in NGA buffer (5mM EDTA, 150mM NaCl, 50mM Tris base, 10mM NaN_3, 0.25% gelatin, and 0.05% Nonidet P40). The blots were then rinsed in saline and incubated at 4^{o}C for a further 16hr with a polyclonal rabbit anti-human topoisomerase II IgG antibody (diluted 1:1000 in NGA buffer) kindly supplied by Dr Leroy Liu (Johns Hopkins Oncology Center, Baltimore MD). The nitrocellulose sheet was then washed in saline and incubated with second (indicator) antibody, ^{125}I-labelled swine anti-rabbit IgG, for 16hr at 4^{o}C; autoradiographic exposure (24hr) was then used to visualise antibody binding to topoisomerase II.

RESULTS AND DISCUSSION

Lack of correlation between DNA cleavage and DNA synthesis

Control experiments confirmed that the enhancement of VP-16-induced DNA cleavage seen with oestrogen exposure was antagonised by the topoisomerase II inhibitor novobiocin, as reported previously (10). No such antagonism was seen using the ribonucleotide reductase inhibitor hydroxyurea (Table 1), however, despite induction of cell-cycle arrest at the G_1/S interface documented by flow cytometry (data not shown). This is consistent with the observation reported by ourselves (11) and others (16) that topoisomerase-II-mediated DNA cleavage is not inhibited by aphidicolin, and strongly suggests that such cleavage does not depend

Table 1 Attenuation of VP-16-induced DNA cleavage by various inhibitors

	DNA cleavage (%)	
	Control	Oestrogen-treated
Novobiocin	103.2 ± 25.1	34.8 ± 16.2
Hydroxyurea	90.8 ± 15.1	96.0 ± 8.0
3-aminobenzamide	99.3 ± 9.0	92.2 ± 4.4

Oestrogen (10^{-8}M), ethanol (0.1%; in controls), and hydroxyurea (2mM) were applied for 24hr; novobiocin (1mM) was applied for one hour prior to VP-16 treatment, and maintained during 60 min drug exposure. Results are based on measurements of DNA cleavage at three different VP-16 concentrations.

upon active DNA replication. VP-16-induced DNA cleavage was similarly unaffected by 3-aminobenzamide (Table 1), thus tending to exclude poly(ADP)ribosylation as an activating mechanism for topoisomerase II in this cell system.

DNA cleavage induced by the topoisomerase-II-interactive drug **m**-AMSA (17) was dramatically enhanced within 4hr of oestrogen exposure (Fig. 1a), a finding in agreement with the time-course previously reported for enhancement of VP-16-induced DNA cleavage in this cell system (11). Since DNA synthesis was only measurably enhanced after 8-16hr oestrogen stimulation in the latter series of parallel experiments (11), this further suggests that oestrogen enhances the

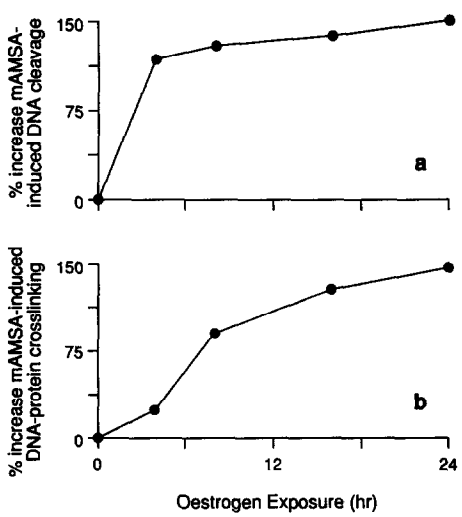

Figure 1 Temporal sequence of **m**-AMSA-induced events following oestrogen stimulation. (a), m-AMSA-induced DNA cleavage measured by alkaline unwinding. (b), Filter binding of DNA-protein complexes crosslinked by m-AMSA.

Table 2 Duration of oestrogen-induced DNA cleavage enhancement

	Duration of oestrogen pre-treatment (hr)			
	24	72	120	168
DNA cleavage enhancement (%)	108.3	81.3	50.4	0.9
Cell growth enhancement [T_D(DC)/ T_D(E2)]	1.43 ±0.14	2.13 ±0.44	2.68 ±0.69	2.72 ±0.68

T-47D cells were treated with 5uM VP-16 at the indicated times after commencement of oestrogen stimulation. The percentage DNA cleavage enhancement (E) was calculated from the expression, E = (fe - fc)/fc, where fe and fc represent DNA cleavage induced in oestrogen-treated and control cells respectively. Results are based on two experiments. Population doubling times (T_D) were calculated on the basis of cell counts performed 24-48hr before and after each respective oestrogen exposure time, and are based on four experiments.

enzyme:DNA interaction in cells not yet committed to active replication, i.e. in G_1-phase cells.

To assess the more long-term relationship between drug-induced DNA cleavage and DNA synthesis, the extent of VP-16-induced cleavage was ascertained in cells exposed to oestrogen for up to one week; sustained growth enhancement is seen in T-47D cells exposed to oestrogen for this duration (11). Table 2 shows that enhancement of VP-16-induced DNA cleavage declines progressively after 24-72hr oestrogen exposure. This finding implies that the observed enhancement represents a transient response to oestrogen stimulation in T-47D cells, and re-emphasises the lack of correlation with DNA synthesis.

DNA-protein crosslinking studies

In order to clarify the significance of the DNA cleavage results, extractable topoisomerase II was measured using m-AMSA-induced DNA-protein crosslinking as validated by Minford *et al* (13). These experiments confirmed that enhanced DNA-protein crosslinking is readily observable within 4-8hr of oestrogen exposure (Fig. 1b). Again, the time-course of this enhancement is in contrast to that already reported for tritiated thymidine incorporation (11).

Correlation of DNA cleavage with topoisomerase II synthesis

In an earlier report we showed that cycloheximide antagonised the enhancement of VP-16-induced DNA cleavage seen in oestrogen-stimulated T-47D cells (11). This

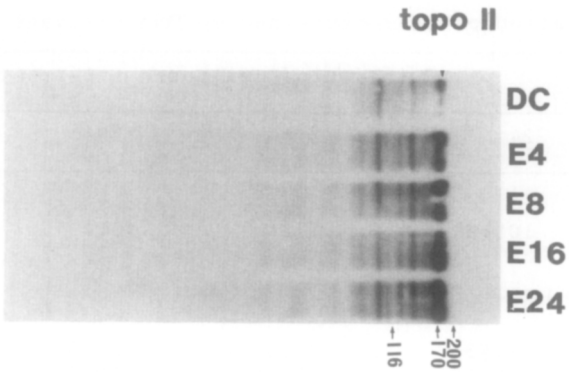

Figure 2 Cellular topoisomerase II content measured by immunoblotting at various intervals following oestrogen exposure. DC, control cells maintained in medium containing dextran-coated charcoal-stripped serum without added oestrogen; E4, cells exposed to oestrogen for 4hr; E8, oestrogen 8hr; E16, oestrogen 16hr; E24, oestrogen 24hr.

suggested that the observed DNA cleavage enhancement was dependent upon protein synthesis, but did not establish whether the protein in question was topoisomerase II itself or, alternatively, a molecule which activates it. Western blotting confirms that the enzyme is indeed synthesised within 4hr of oestrogen stimulation (Fig. 2). When considered in the context of the cycloheximide (11) and 3-aminobenzamide data, this result strongly suggests that enhancement of topoisomerase II synthesis occurs as an early response to oestrogen exposure in T-47D cells, and that this enhancement is quantitatively related to the recognised increases in drug-induced DNA cleavage (11) and cytotoxicity (10).

CONCLUSION

In this study we have demonstrated that T-47D cells synthesise topoisomerase II as an early (and probably transient) response to oestrogen stimulation, and that this increase in cellular topoisomerase II content occurs independently of DNA synthesis. Taking into account our previous observation of a biphasic dose-dependent enhancement of VP-16 cytotoxicity by oestrogen (10), these experiments suggest that oestrogen recruits a G_1-phase cell subset characterised by elevated topoisomerase II content. Further studies will be required to clarify whether this implies a major role for topoisomerase II in transcriptional activation of T-47D cells.

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