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Inhibition of Repair Patch Ligation by an Inhibitor of Poly(ADP-ribose) Synthesis in Normal Human Fibroblasts Damaged with Ultraviolet Radiation

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SUMMARY

The effect of inhibiting poly(ADP-ribose) synthesis on DNA excision repair following UV irradiation of cultured normal human fibroblasts was determined under conditions which did not perturb NAD^+ concentration. Following UV irradiation, there was a transient increase in DNA strand breaks to a maximum of 800 rad eq of breaks 30 min after damage. 3-Aminobenzamide (5 mM) caused a 50% increase in the maximum number of DNA single strand breaks following damage but did not prevent the decline in strand breaks which normally occurs within the first hour after damage. Addition of 3-aminobenzamide several hours after damage, when most of the strand breaks had disappeared, caused a reaccumulation of strand breaks. 3-Aminobenzamide inhibited ligation of repair patches, as measured by exonuclease III, following damage by UV radiation and the magnitude of the inhibition was sufficient to account for the increases in strand breaks caused by 3-aminobenzamide. UV radiation alone did not lower NAD^+ concentrations; however, when the repair synthesis step was inhibited by aphidicolin and hydroxyurea, the number of single strand breaks increased and the NAD^+ concentration fell to 11%. 3-Aminobenzamide inhibited this depletion of NAD^+ by 80%.

Poly(ADP-ribose) is a nuclear polymer synthesized from NAD^+ in response to DNA strand breaks. Although many possible roles have been proposed for poly(ADP-ribose), most studies have focused on a possible role in DNA excision repair (for reviews, see Refs. 1–3). It is well established that ionizing radiation and alkylating agents stimulate the synthesis of poly(ADP-ribose) and, at high doses, result in a depletion of NAD^+ (4–7). Furthermore, inhibitors of poly(ADP-ribose) synthesis, such as 3-ABA, inhibit the repair of DNA damage from ionizing radiation and alkylating agents (8–13). In the case of damage from UV radiation, it has been found that strand breaks created during the repair process can stimulate poly(ADP-ribose) synthesis (14, 15); however, to date, no study has detected an inhibitory effect of 3-ABA on excision repair following UV radiation (10–12, 15–19).

In three recent studies, inhibitors of DNA repair synthesis, such as aphidicolin, arabinofuranosylcytosine, and hydroxyurea, were used to accumulate incised sites to high levels. The repair synthesis inhibitors were then removed and the effect of an inhibitor of poly(ADP-ribose) synthesis on either the disappearance of strand breaks (15, 16) or on the ligation of repair patches, measured by Exo III (20), was determined. In one of these studies, it was found that 3-ABA actually stimulated ligation of repair patches as measured by Exo III (20). The obvious potential limitation of the experimental approaches described above is that the drug-induced accumulation of large numbers of strand breaks will stimulate poly(ADP-ribose) which could result in a depletion of NAD^+, which in turn would inhibit poly(ADP-ribose) synthesis. Obviously, these would not be appropriate conditions under which to determine the effects of an inhibitor of poly(ADP-ribose) synthesis. Unfortunately, NAD^+ concentrations were not measured in the studies mentioned above, and, therefore, one cannot be sure if this is in fact a defect in these experimental approaches. Furthermore, many studies of poly(ADP-ribose) metabolism following damage by alkylating agents have used relatively high doses which may also have depleted NAD^+ concentrations (8–13). Clearly, there is a need both to examine the validity of experimental approaches which involve the generation of large numbers of strand breaks and to determine the effects of inhibitors of poly(ADP-ribose) synthesis on DNA repair under conditions which have not perturbed NAD^+ concentrations.

In the present study, we determined the effect of an inhibitor

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ABBREVIATIONS: ABA, 3-aminobenzamide; Exo III, exonuclease III; MNU, *N*-methyl-*N*-nitrosourea; PBS, phosphate-buffered saline; EDTA, ethylenediaminetetraacetate.

of poly(ADP-ribose) synthesis on excision repair following UV irradiation under conditions which did not perturb NAD^+ concentrations. The effect of 3-ABA on the relative rates of incision and ligation, as reflected by strand break frequencies, was determined at early and late times after damage. As well, the effect of 3-ABA on repair patch ligation, measured using Exo III, was determined. Finally, we tested whether accumulating large numbers of strand breaks using inhibitors of repair synthesis could result in a depletion of NAD^+, and whether this depletion could be prevented by 3-ABA.

Materials and Methods

Cell culture. Human diploid fibroblasts (AG1518; Institute for Medical Research) were subcultured into plastic tissue culture plates, prelabeled with [^{14}C]dThd (Amersham Corp., 50–60 mCi/mmol, 15 nCi/ml) for 1 week, and grown to confluence. Cell integrity was quantitated as follows. Cells were rinsed with PBS and incubated with a 0.5% solution of trypan blue in PBS (Flow Laboratories) for 10 min at room temperature; then, the percentage of cells not stained with trypan blue was determined.

Damaging of cells with UV radiation. Cells were damaged with UV radiation by removing the medium from the culture plate and exposing the cells to radiation from a G15T8 germicidal lamp at a flux of 1.2 W/m^2. UV fluxes were measured using an Ultraviolet Products ultraviolet meter.

Measurement of DNA strand breaks. DNA strand breaks were measured using a modified version of the alkaline elution method of Kohn *et al.* (21). Fibroblasts (~2 × 10^6 cells), prelabeled with [^{14}C]dThd, were damaged and incubated as described, and then harvested by gentle scraping in 2 ml of ice-cold PBS containing 0.02% EDTA. One ml of cell suspension was added to 20 ml of ice-cold PBS containing an internal standard consisting of ~5 × 10^5 L1210 cells which had been labeled for ~16 hr with 25 nCi/ml [^{3}H]dThd and irradiated with 200, 1000, or 2000 rad of γ-radiation, depending on the required range of the assay. The cells were collected on a polycarbonate filter (Nuclepore Corp., Pleasanton, CA), washed with 5 ml of PBS, lysed with 4 ml of 2 M NaCl, 40 mM EDTA, 0.2% *N*-laurylsarcosine, pH 10. This was followed by 5 ml of 20 mM EDTA, pH 10, and then the DNA was eluted with 20 mM EDTA adjusted to pH 12.0 with tetrapropylammonium hydroxide, at a flow rate of 3 ml/100 min, 20 ml/100 min, or 50 ml/100 min for the 200, 1000, and 2000 rad assays, respectively. The radioactivity of the eluted DNA was determined by liquid scintillation counting. Standard curves were constructed by irradiating fibroblast monolayers with γ-rays so that data could be expressed in rad eq.

Measurement of repair ligation using Exo III. Ligation of repair patches was measured essentially as described previously (22). The assay is based on the fact that unligated repair patches should have free 3′-hydroxyl ends and therefore will be sensitive to Exo III, a 3′-5′ exonuclease. DNA was isolated by addition of proteinase K (0.1 mg/ml) in 200 mM Tris (pH 8.5 at 20°), 1% sodium dodecyl sulfate, 100 mM EDTA to cells, followed by incubation at 47° for 2 h. The samples were extracted three times with phenol and three times with isoamyl alcohol/chloroform (1:24). The DNA was precipitated with ethanol and dissolved in Exo III digestion buffer (50 mM Tris, pH 8.0 at 37°, 5 mM $MgCl_2$, 10 mM β-mercaptoethanol, 0.5 mg/ml nuclease-free bovine serum albumin) overnight, at 4° on a rotating wheel. All manipulations of the DNA were designed to minimize shearing. The DNA was digested at 37° with 0.2 unit of Exo III (Bethesda Research Laboratories, Gaithersburg, MD) per μg of DNA and samples were removed at several times during the 50-min digestion period for determination of perchloric acid-soluble ^{3}H (repair label) and ^{14}C (bulk label) radioactivity expressed as a fraction of the total radioactivity in the DNA being digested.

Measurement of the NAD^+ content of cells. Fibroblast monolayers (~2 × 10^6 cells) were extracted with ice-cold 0.4 M perchloric acid at 4° for 20 min. The perchloric acid was neutralized by extraction with a 0.5 M solution of Alamine 336 (tricaprylyl tertiary amine; a gift from Henkel Corp., Minneapolis MN) in Freon-TF (Dupont, Maitland, Ontario), and an aliquot of the sample was used to determine the NAD^+ content by high performance liquid chromatography, using a Whatman ODS-3 reversed phase column (Whatman Inc., Clifton, NJ), eluted with a gradient of 100% 0.1 M ammonium phosphate, pH 5.1, to 86.5% 0.1 M ammonium phosphate, pH 5.1, and 7.5% methanol, as described previously (23). Detection was by absorbance at 254 nm. This method measures only the oxidized form of NAD.

Results

Effect of 3-ABA on the relative rates of incision and repair patch ligation under conditions of normal NAD^+ concentration. Following UV irradiation of cells, strand breaks are generated by the incision step of excision repair and removed by the ligation step; therefore, any treatment which results in a reduction in the rate of ligation relative to incision will result in an increase in strand breaks. To determine the effect of 3-ABA on the relative rates of incision and ligation, confluent normal human fibroblasts were damaged with 10 J/m^2 of UV radiation and incubated with or without 3-ABA (5 mM), an inhibitor of poly(ADP-ribose) synthetase (24). These treatments had no effect on NAD^+ concentration (see, for example, Table 3). The curves relating DNA strand breaks to time after damage (Fig. 1) consist of two phases: an early phase in which the rate of incision exceeded the rate of ligation such that strand breaks accumulated, and a late phase in which the rate of ligation exceeded the rate of incision such that the number of strand breaks declined. 3-ABA caused a 50% increase in the maximum number of DNA strand breaks but, interestingly, did not prevent the decline in strand break frequency at later times, suggesting that poly(ADP-ribose) may only be required at early times of repair.

The involvement of poly(ADP-ribose) in excision repair at intermediate and late times following UV irradiation. Given that poly(ADP-ribose) undergoes only a transient accumulation following damage with ionizing radiation and alkylating agents, that few strand breaks are present at late times following UV radiation, and that 3-ABA only increased strand break frequencies but did not prevent the disappearance of strand breaks following UV irradiation (see Fig. 1), the possibility was considered that poly(ADP-ribose) might be involved in repair only at early times following damage of DNA. If the turnover of strand breaks is sufficiently rapid at late times following UV irradiation, then the strand break frequency will be very sensitive to a reduction in the rate of ligation. Aphidicolin, an inhibitor of DNA polymerases α and δ, was used to determine the rate of strand break turnover (25). As shown in Table 1, a 15-min incubation with aphidicolin 6 or 24 hr after UV irradiation resulted in a rapid accumulation of strand breaks, indicating that the low level of strand breaks does not indicate a low level of repair activity but rather a steady state situation in which the rates of incision and ligation are equal. Although aphidicolin may not have completely inhibited repair patch completion, one can estimate the minimum rates of both incision and ligation to be 2200 rad eq/hr (i.e., ~6600 sites/hr) 4-hr after damage, and 800 rad eq/hr (~2400 sites/hr) 24 hr after damage. Addition of 3-ABA to cells 4 or 22 hr after UV irradiation resulted in a reaccumulation of DNA strand breaks; however, aphidicolin was much more effective than 3-ABA at increasing strand break frequencies.

Repair patch ligation. One difficulty in interpreting DNA

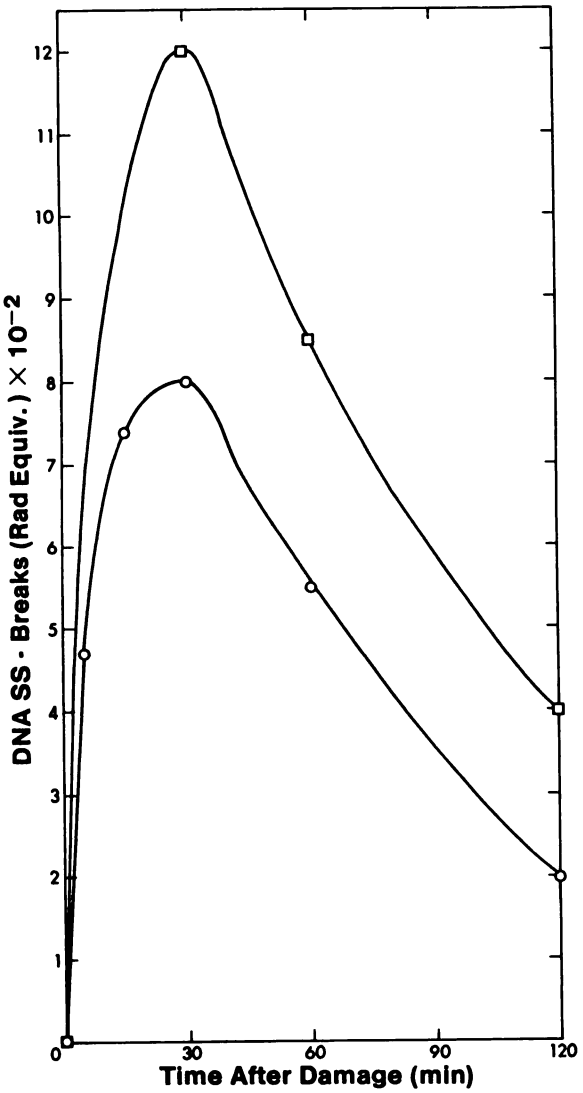

Fig. 1. The effect of 3-ABA on the number of DNA strand breaks following treatment of cells with 10 J/m² UV radiation. Confluent normal human fibroblasts were incubated for 30 min at 37° with or without 5 mM 3-ABA, damaged with 10 J/m² UV radiation, and incubated at 37° in the presence or absence of 5 mM 3-ABA. DNA strand breaks were determined by alkaline elution (Materials and Methods). ○, control; □, 3-ABA.

strand break data is that an increase in strand breaks could result from a stimulation of incision or an inhibition of ligation (see Fig. 1 and Table 1). In fact, in order to account for the inhibition by 3-ABA on the disappearance of strand breaks following damage by alkylating agents, it has been proposed that 3-ABA stimulates incision (13, 20). Therefore, in order to quantitate the effect of an inhibition of poly(ADP-ribose) synthesis on repair patch ligation, Exo III was used to determine the fraction of repair-incorporated nucleotides present in unligated patches (see Materials and Methods). When cells were damaged with UV radiation and incubated with [³H]dThd to label repair patches, 90% of repair-incorporated nucleotides were present in ligated patches after a 30-min incubation (Table 2). 3-ABA (5 mM) inhibited ligation but was not as potent as hydroxyurea (10 mM), an indirect inhibitor of repair synthesis.

Depletion of NAD⁺ by strand breaks generated during excision repair. The use of inhibitors of repair synthesis, such as aphidicolin, to accumulate strand breaks to high levels in studies of the role of poly(ADP-ribose) in excision repair assumes that these conditions do not inhibit poly(ADP-ribose) synthesis through depletion of NAD⁺. To test this assumption, we determined the ability of the incised sites and unligated repair patches produced during UV-induced excision repair to deplete NAD⁺. As shown in Table 3, 10 J/m² UV radiation resulted in a peak of 800 rad eq of strand breaks 30 min after damage but had no effect on the NAD⁺ concentration. Addition

TABLE 1

The effect of 3-ABA and aphidicolin on DNA strand breaks at intermediate and late times following irradiation of cells

Confluent normal human fibroblasts were damaged with UV radiation (10 J/m²) and then incubated at 37°. 3-ABA (5 mM) or aphidicolin (2.5 μg/ml) was added at the times indicated. The cells were washed and harvested in ice-cold PBS and strand break frequencies were determined by alkaline elution (Materials and Methods). Neither aphidicolin nor 3-ABA induced DNA strand breaks in undamaged cells. Cell integrity, as determined by trypan blue exclusion in a separate experiment (Materials and Methods), was greater than 90% under all experimental conditions.

Inhibitors	Time of addition of drugs	DNA strand breaks	Drug-induced increase in strand breaks
	hr after damage		*rad eq*
None	4	170	
None	6	120	
3-ABA, 2 hr	4	250	130
Aphidicolin, 0.25 hr	5.75	660	540
None	24	0	
3-ABA, 2 hr	22	50	50
Aphidicolin, 0.25 hr	23.75	200	200

TABLE 2

The effect of 3-ABA and hydroxyurea on ligation of DNA repair patches following UV radiation

Confluent normal human fibroblasts were damaged with UV radiation (10 J/m²) and then incubated 30 min at 37° with [³H]dTHd in the presence or absence of 3-ABA (5 mM) or hydroxyurea (10 mM), and harvested in ice-cold PBS. The percentage of repair-incorporated nucleotides (³H-labeled) present in unligated DNA repair patches was determined by digesting purified DNA from the cells with Exo III (Materials and Methods).

DNA damaging agent	Inhibitors	% of Repair-incorporated nucleotides present in unligated patches after 30-min pulse with [³H]dThd
UV (10 J/m²)	None	10
	3-ABA	27
	Hydroxyurea	46

TABLE 3

DNA single-strand breaks and NAD⁺ concentration following damage of AG1518 human fibroblasts by UV radiation

Confluent normal human fibroblasts were damaged with UV radiation (10 J/m²) and then incubated at 37° for the times indicated in the presence or absence of hydroxyurea (10 mM) and aphidicolin (2.5 μg/ml). Duplicate monolayer cultures were used for each time point: one culture was extracted with perchloric acid for the determination of NAD⁺ while the other culture was used for the DNA strand break assay.

Inhibitors	Time after damage	DNA single-strand breaks	NAD concentration
	min	*rad eq*	*% of control*
	30	880	90
	120	200	103
Hydroxyurea + aphidicolin	30	2500	100
Hydroxyurea + aphidicolin	60	>3000	40
Hydroxyurea + aphidicolin	120	>3000	11

of hydroxyurea and aphidicolin to UV-treated cells increased the number of strand breaks and caused a time-dependent depletion of NAD^+.

Inhibition of NAD^+ depletion by 3-ABA. 3-ABA was used to test whether the depletion of NAD^+ induced by UV radiation resulted from the stimulation of poly(ADP-ribose) synthesis. Treatment of cells with UV radiation alone (10 J/m^2) had no effect on NAD^+, whereas UV radiation plus aphidicolin (2.5 μg/ml) reduced NAD^+ concentration to 14% of control after 2 hr (Table 4). 3-ABA (5 mM) prevented approximately 80% of the NAD^+ depletion, suggesting both that the depletion results from the synthesis of poly(ADP-ribose), and that poly(ADP-ribose) synthesis is substantially inhibited by 3-ABA under the conditions used in the present study.

Discussion

Following treatment of cells with UV radiation, there was a transient increase in the number of DNA strand breaks (Fig. 1), as previously reported (26). This "burst" of strand breaks may be a synchrony effect resulting from the fact that incision occurs rapidly immediately after damage but ligation cannot begin until repair patch synthesis has occurred. Strand break data following UV radiation are relatively simple to interpret because all the breaks are created by incision and removed by ligation (i.e., there are no detectable alkali-labile sites or frank breaks as are created by alkylating agents and ionizing radiation; see Fig. 1). Thus, the increase in strand breaks caused by 3-ABA is consistent with a stimulation of incision or an inhibition of ligation (Fig. 1). In contrast to our results, Collins (16) observed a reduction in strand break frequencies by 3-ABA following UV irradiation; however, those experiments were carried out in the presence of hydroxyurea and arabinofuranosylcytosine. Cleaver *et al.* (11) observed no effect of 3-ABA on strand breaks during a 2-hr incubation following UV irradiation of human cells. In contrast, we observed 2-fold more breaks in 3-ABA-treated cells 2 hr after irradiation (Fig. 1).

The results presented in Fig. 1 and Table 1 are in apparent disagreement with those of Synder (17), who used a nick-translation assay in permeable cells to determine the effects of drugs on the frequency of free 3′-hydroxyl ends in DNA. Treatment of UV-irradiated cells with drugs such as hydroxyurea and aphidicolin stimulated subsequent incorporation of radioactive nucleotides by DNA polymerase I, but 3-ABA had no effect. A possible explanation for the apparent discrepancy between Synder's results (17) and ours is that the unligated repair patches generated by 3-ABA may not be readily accessible to DNA polymerase I in the chromatin of permeable cells.

We considered the possibility that poly(ADP-ribose) might only be involved at early times after damage; however, the results in Table 1 show that 3-ABA caused strand break reaccumulation both at 4 and 22 hr after damage with UV, consistent with a stimulation of incision or an inhibition of ligation. The rapid reaccumulation of strand breaks induced by aphidicolin demonstrates that the low level of strand breaks at late times of repair are the result of a steady state in which the rates of incision and ligation are equal. Under steady state conditions the number of repair patches in progress (i.e., undergoing synthesis or ligation) will equal the number of strand breaks. Therefore, assuming a value of ~3 strand breaks/genome/rad eq of breaks (21), the number of patches in progress 6 hr after damage with UV was ~360/cell (Table 1), and must have been less than the lower limit of detection (~60 breaks/cell) 24 hr after damage, since no strand breaks were detected at this time. Since the rate of strand break formation in the presence of aphidicolin gives a minimum estimate for the rate of incision of 6600 sites/hr/cell 6 hr after damage, one can calculate the time required to complete a repair patch to be ~3 min, a value in agreement with previous estimates of 3–10 min and 4 min (22, 26).

The direct measurement of the state of ligation of repair patches using Exo III demonstrated that 3-ABA inhibited the ligation of repair patches following damage with UV (Table 2). This approach still does not allow one to determine the precise cause of the inhibition of ligation since it could have occurred at either the level of repair synthesis or ligation. Although the extent of inhibition of ligation was not large, it is probably sufficient to account for the effect of 3-ABA on strand break frequencies, thus suggesting that 3-ABA does not stimulate incision.

It has been hypothesized that poly(ADP-ribose) synthesis may constitute a suicide response of cells to very high levels of DNA damage which would involve the depletion of NAD^+ and, consequently, the depletion of ATP, thus resulting in cell death. In the present study, even conditions which depleted NAD^+ by ~90% had no effect on ATP (data not shown). Jacobsen *et al.* (27) observed no reduction of ATP concentration in 3T3 cells damaged with an alkylating agent under conditions which reduced NAD to 50% of control, and Hunting *et al.* (23) observed no effect on ATP in L1210 cells treated with MNU in which NAD concentration was 32% of control (23). These data suggest that depletion of NAD^+ alone is not sufficient to induce a depletion of ATP. It has also been proposed that the stimulation of repair synthesis by inhibitors of poly(ADP-ribose) synthesis results from the preservation of nucleotide pools (28); however, this would not explain the 2-fold stimulation of repair synthesis by nicotinamide in permeable cells in which nucleotides were supplied exogenously (29).

We determined the ability of incised sites and unligated repair patches formed during UV-induced repair to reduce NAD^+ concentration (Table 4). Strand breaks formed during the repair of UV damage were able to induce a depletion of NAD^+ but only if they were accumulated to high levels by incubation of cells with inhibitors of repair synthesis, consistent with previous studies (14, 15). This suggests that the recent experimental approach involving the accumulation of strand

TABLE 4

The effect of 3-ABA on the UV/aphidicolin-induced depletion of NAD^+

Confluent normal human fibroblasts, either damaged or undamaged, were incubated for a total of 2 hr at 37° with or without 3-ABA (5 mM) and/or aphidicolin (2.5 μg/ml). The cells were then extracted with perchloric acid on ice and NAD^+ concentrations were determined (Materials and Methods).

Treatment	Inhibitors	Time after damage	NAD^+ concentration
		hr	*% of control*
Control	5 mM 3-ABA	2	115
	Aphidicolin	2	120
	3-ABA + aphidicolin	2	100
UV (10 J/m^2)	None	2	103
	Aphidicolin	2	14
	3-ABA + aphidicolin	2	82

breaks to high levels using inhibitors of repair synthesis in order to determine the effect of 3-ABA on repair may give misleading results since poly(ADP-ribose) synthesis may already have been inhibited by the depletion of NAD^+. Our finding that 3-ABA prevented the depletion of NAD^+ supports the suggestion by Cleaver and Park (20) that the stimulation of ligation by 3-ABA they observed may have resulted from the preservation of NAD^+ concentration. This stimulation of ligation by 3-ABA suggests that it has no direct inhibitory effects on repair synthesis or ligation.

Finally, our results show that, although 3-ABA clearly inhibits repair patch ligation, the extent of inhibition of repair is much less than the inhibition of poly(ADP-ribose) synthesis, as measured by prevention of NAD^+ depletion. A trivial explanation for these results is that the inhibition of repair by 3-ABA is unrelated to the inhibition of poly(ADP-ribose) synthesis, as previously suggested (for review, see Ref. 3). A second, more interesting possibility is that poly(ADP-ribose) synthesis is not an essential step in every repair event. For example, it might facilitate repair in certain inaccessible regions of chromatin.

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