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Inhibition of poly(ADP-ribose) polymerase activity affects its subcellular localization and DNA strand break rejoining

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Poly(ADP-ribose) polymerase (PARP) plays a crucial role in DNA repair. Modulation of its activity by stimulation or inhibition is considered as a potentially important strategy in clinical practice, especially to sensitize tumor cells to chemo- and radiotherapy through inhibition of DNA repair. Here we studied the effect of the three PARP inhibitors, 5-iodo-6-amino-benzopyrone (INH_2BP), 1,5-isoquinolinediol (1,5-dihydroxyisoquinolinediol (1,5-IQD) and 8-hydroxy-2-methylquinazolin-4-[3*H*]one (NU1025), and for two of them the efficiency in slowing the rejoining of DNA strand breaks induced by H_2O_2 was compared. Inhibition of PARP changed its intranuclear localization markedly; cells exposed to the inhibitor NU1025 showed a significant tendency to accumulate PARP in large foci, whereas in untreated cells its distribution was more uniform. The speed and efficiency of rejoining of H_2O_2-induced DNA strand breaks were lower in cells incubated with a PARP inhibitor, and the kinetics of rejoining were modulated in a different manner by each inhibitor. At a concentration of 100 μM the efficiency of the inhibitors could be ranked in the order NU1025>IQD>INH_2BP. The two first compounds were able to decrease the overall PARP activity below the level detected in control cells, while INH_2BP showed up to 40% PARP activity after exposure to H_2O_2.

Keywords: poly(ADP-ribose) polymerase (PARP), PARP inhibitors, PARP foci, efficiency of PARP inhibition, DNA strand break rejoining

INTRODUCTION

Poly(ADP-ribose) polymerase (PARP), an enzyme which catalyses the synthesis of long branched homopolymers of ADP-ribose (poly(ADP) ribose, PAR) from molecules of NAD^+, is among the most abundant proteins in cell nuclei. It carries out reversible post-translational poly(ADP-ribosyl)ation of proteins (reviewed in: D'Amours *et al.*, 1999; Huber *et al.*, 2004; Petermann *et al.*, 2005) and has multiple functions from DNA repair and transcription to cell survival and death (reviewed in: D'Amours *et al.*, 1999; Shall & de Murcia, 2000; Herceg & Wang, 2001; Virag & Szabo, 2002; Petermann *et al.*, 2005). PARP is a component of numerous multiprotein complexes including the DNA base excision repair (BER) machinery; DNA damage is the most important signal for poly(ADP-ribosylation) reactions (D'Amours *et al.*, 1999; Bürkle, 2005) and PARP recognizes and binds to DNA strand breaks generated by genotoxic agents. Despite many studies in this field, the precise role of PARP in BER is still unclear; it is known to control the synchronization of repair enzymes (Allinson *et al.*, 2004) and has been proposed to sig-

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Abbreviations: BER, base excision repair; INH_2BP, 5-iodo-6-amino-benzopyrone; 1,5-IQD, 1,5-isoquinolinediol (1,5-dihydroxyisoquinolinediol); NU1025, 8-hydroxy-2-methylquinazolin-4-[3*H*]one; PARP, poly(ADP-ribose) polymerase; PAR, poly(ADP) ribose; PBS, phosphate-buffered saline.

nal DNA damage and to dissociate histones, relax chromatin, and recruit repair enzymes at damage sites and to operate as a modulator of BER capacity (D'Amours *et al.*, 1999; Bürkle, 2005; Malanga & Althaus, 2005; Woodhouse & Dianov, 2008).

PARP is thus a potential target for stimulating or inhibiting DNA repair, and modulation of (ADP-ribosyl)ation is considered a promising approach in clinical practice (Pero *et al.*, 1995; Miwa & Masutani, 2007). We recently reported that transient stimulation of PAR synthesis by a non-genotoxic agent increases the speed and efficiency of DNA strand break rejoining in cells after oxidative stress (Ryabokon *et al.*, 2008). On the other hand, inhibition of PARP prevents cells from drastic production of poly(ADP-ribose) following DNA damage, and hence from depletion of their NAD^+ pool with consequent energy deprivation and necrosis (reviewed in Virag & Szabo, 2002). By reducing DNA repair, PARP inhibition can lead to cell death, this suggests a promising approach to sensitize tumor cells to chemo- and radiotherapy (Cosi, 2002; Haince *et al.*, 2005) and several inhibitors of PARP including NU1025 are being evaluated in clinical trials to potentiate the genotoxic action of chemo- or radiotherapy for treatment of cancers, as well as of neurodegenerative diseases and ischemia reperfusion-induced tissue injuries (reviewed in Haince *et al.*, 2005). However, there are only limited data on the effects of PARP inhibitors on DNA repair and its kinetics. 3-Aminobenzamide (3AB), one of the first-generation inhibitors, reduced the rate of DNA strand break rejoining in mouse, human, and Chinese hamster cells following the DNA-damaging action of γ-irradiation, dimethyl sulfate, methylnitrosourea, or a hydroperoxide analogue (Shall, 1984; Palomba *et al.*, 2001). NU1025 showed a similar effect and increased the level of DNA single and double strand breaks in CHO-K1 cells after ionizing irradiation in a concentration-dependent manner (Boulton *et al.*, 1999).

The objectives of the present study were to examine the efficiency of the second generation PARP inhibitors 5-iodo-6-amino-benzopyrone (INH_2BP), 1,5-isoquinolinediol (1,5-dihydroxy-isoquinolinediol (1,5-IQD), and 8-hydroxy-2-methylquinazolin-4-[3*H*]-one (NU1025) in inhibiting PARP and modulating DNA strand break rejoining in human cells after acute genotoxic damage. We found that inhibition of PARP activity has a profound effect on its intracellular localization and on the kinetics of DNA strand break rejoining in cells exposed to H_2O_2. The PARP inhibitors studied showed different efficiencies and modulated DNA strand break rejoining with different kinetics. We further observed that dimethyl sulfoxide (DMSO), a commonly used solvent for PARP inhibitors which has radical-scavenging properties, may mask the capacities of the inhibitors to suppress DNA repair.

MATERIALS AND METHODS

PARP inhibitors. 5-Iodo-6-amino-benzopyrone (INH_2BP) and 1,5-isoquinolinediol (1,5-dihydroxyisoquinolinediol, 1,5-IQD) (Calbiochem) were kindly provided by R. Hancock (Laval University Cancer Research Center, Québec, Canada) and 8-hydroxy-2-methylquinazolin-4-[3*H*]one (NU1025) was from Sigma (Sigma-Aldrich, St. Louis, USA). These compounds were dissolved in DMSO at 50 mM and aliquots were stored in the dark at –20°C.

Cell culture and exposure to H_2O_2. Raji cells (human B-lymphoblastic leukemia) and K562 cells (human myelogenous leukaemia) were cultured in RPMI 1640 with L-glutamine (Sigma-Aldrich), 15% fetal bovine serum (FBS; Gibco) and 0.04% gentamycin at 37°C in a humidified atmosphere and 5% CO_2. Exponentially growing cells were used in all experiments after washing in ice-cold PBS. Cells were incubated in growth medium with or without a PARP inhibitor (100 μM) at 4°C for 10 min and then with 100 μM H_2O_2 for 1 min to induce DNA damage. After washing twice with ice-cold PBS for 8–10 min the cells were incubated in growth medium at 37°C, supplemented or not with the same PARP inhibitor at 100 μM.

Localization and quantification of PARP. Detection and quantification were as described previously (Ryabokon *et al.*, 2008). Briefly, cells were cytospun onto glass slides at 4°C, fixed in methanol/acetone (1:1) at –20°C, and incubated in 5% non-fat milk:PBS:0.05% Tween-20 to prevent non-specific antibody binding. PAR was immunolabeled with rabbit polyclonal antibody LP96-10-04 and PARP-1 with mouse monoclonal antibody (C2-10) (Alexis, 1:50 dilution). PAR was then visualized with a highly cross-absorbed Alexa Fluor 488-labeled goat anti-rabbit IgG (Invitrogen-Molecular Probes, 1:100 dilution) and PARP with Alexa Fluor 568-labeled goat anti-mouse IgG (H+L) (Invitrogen-Molecular Probes, 1:100 dilution). Nuclei were stained with DAPI (1 μg/ml). Images were captured with a 40× objective for conventional microscopy or a 63x objective for confocal microscopy (Zeiss LSM 510). The fluorescence intensity of about 200 cells per experimental point was measured using the Lucia 4.60 system (Laboratory Imaging, Prague, Czech Republic) and mean values were expressed as a percentage of the control level (relative intensity, RI).

Measurement of DNA strand break rejoining. The kinetics of DNA strand break rejoining in cells recovering after incubation with H_2O_2 were studied using an alkaline version of the single cell

gel electrophoresis (Comet) assay according to international recommendations (Tice *et al.*, 2000), as described previously (Palyvoda *et al.*, 2002; Ryabokon *et al.*, 2005). All procedures up to fixation of cells in 96% alcohol were performed on ice to avoid additional DNA damage and to stop DNA repair. DNA damage was expressed in arbitrary units (a.u.) (Collins *et al.*, 1993).

Statistical analyses. Means and standard errors of the means of at least 3 replicate experiments were calculated and significance was assessed by the unpaired Student's *t*-test (STATISTICA, StatSoft).

RESULTS

Stimulation of PARP after exposure of cells to H_2O_2

Recently, we reported that detection of PAR in Raji cells by immunofluorescence shows a significant stimulation of its level during 5 min of recovery from a short genotoxic stress with H_2O_2, followed by a gradual decrease during the next 10 min (Ryabokon *et al.*, 2008). Here, the same approach showed a 10-fold increase of PAR in Raji cells 5 min after exposure to H_2O_2 (Fig. 1).

The increase of the level of PAR was smaller when a PARP inhibitor was added at 100 μM during the recovery period; NU1025 showed complete inhibition while 1,5-IQD was less effective (Fig. 1). Nevertheless, these two compounds decreased the PAR level to 15–60% below the control level, showing their ability to inhibit not only the H_2O_2-induced but also the background level of PAR. The third inhibitor, INH_2BP, was the least effective and up to 40% residual PAR remained after exposure to H_2O_2 (Fig. 1).

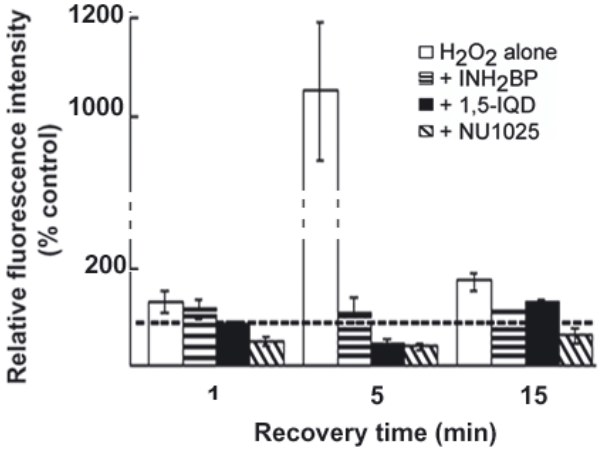

Figure 1. Effect of the PARP inhibitors INH_2BP, 1,5-IQD and NU1025 (100 μM) on the level of PAR measured by immunofluorescence in Raji cells exposed to 100 μM H_2O_2.
Mean values ±S.E. of three experiments are shown. The horizontal dashed line shows the level of PAR in control untreated cells.

Effect of inhibitors of PARP on its distribution in cells exposed to H_2O_2

The distribution of PARP in K562 cells was studied by immunofluorescence at different times during incubation with NU1025 after removal of H_2O_2. PARP accumulated in large foci (Fig. 2). Significantly more cells contained such foci in conditions where PARP was inhibited (Fig. 2B, C), and this effect was more evident at later times of recovery. The average amount of PARP/cell measured for the whole cell population did not change significantly during the experiment.

Effects of PARP inhibitors on DNA strand break rejoining

The most effective inhibitors in our study, NU1025 and 1,5-IQD, were further analyzed as modulators of DNA strand break rejoining. Cells were first incubated in the presence of these inhibitors during 3 h, and no evident genotoxicity of 100 μM solutions of the compounds *per se* or due to inhibition of background activity of PARP was observed (Fig. 3).

In contrast, the PARP inhibitors inhibited the rejoining of DNA strand breaks when they were present before, during, and/or after exposure of cells to H_2O_2 (Fig. 4A). Specifically, 1,5-IQD did not show a significant influence on DNA strand break rejoining during the first minutes of recovery from H_2O_2 but inhibited rejoining in the following 3 h period. The more potent PARP inhibitor NU1025 showed an elevated level of DNA damage from the first minutes of recovery, perhaps due to inhibition of DNA repair in the earlier period including the treatment and washing procedures (Fig. 4A). Thus compounds with different potencies in PARP inhibition modulate the kinetics of DNA strand break rejoining in different manners. Moreover, we found that the complete inhibition of PARP activity following exposure to H_2O_2 combined with reduction of its background activity (Fig. 1) do not completely suppress DNA strand break rejoining.

PARP inhibitors are usually prepared in DMSO as a solvent, although it has antioxidant properties and therefore could reduce DNA damage in cells under genotoxic stress through radical scavenging, thus masking effects of PARP inhibitors in suppressing DNA repair. Our experiments confirmed this hypothesis; the levels of DNA damage in cells pre-, co- and post-incubated with 0.2% DMSO alone were significantly lower than those in cells in-

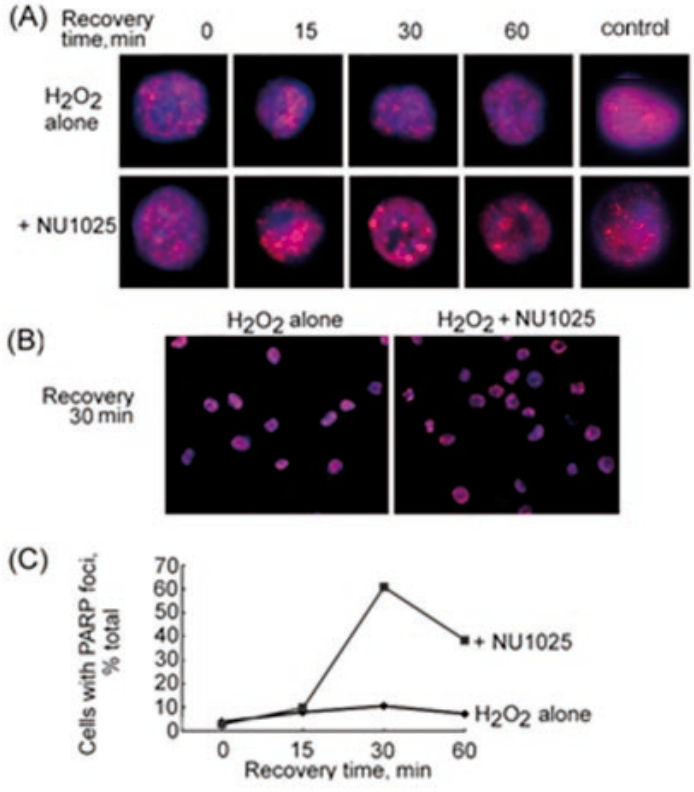

Figure 2. (A and B) Localization of PARP in K562 cells incubated for different times with 100 μM H_2O_2 alone or H_2O_2 and NU1025. (C) frequency of cells with PARP foci with diameter more than 2 μm.

cubated with H_2O_2 only or with H_2O_2 and 100 μM NU1025 in DMSO (Fig. 4B).

DISCUSSION

The relative efficiency of the PARP inhibitors observed here was NU1025, the most potent; IQD with intermediate potency; and INH_2BP the least effective, showing a good correlation with published data (reviewed in Southan & Szabo, 2003). These compounds represent three groups of PARP inhibitors, respectively, benzopyrones, isoquinolinones and quinazolinones. The IC_{50} for INH_2BP and IQD in inhibiting PARP is respectively 10 μM (Southan & Szabo, 2003) and 0.39–1.00 μM (reviewed in Decker *et al.*, 1999). The IC_{50} for NU1025 is 40–500 nM indicating that this inhibitor is at least one order of magnitude more effective than 3-aminobenzamide, one of the first generation inhibitors with an IC_{50} of 2–33 μM (reviewed in Decker *et al.*, 1999). The inhibition of recombinant PARP by 1 μM NU1025 in ELISA tests reached 100%, while that by 1 μM IQD was about 50% (Decker *et al.*, 1999). Our results provide the first direct demonstration that PARP inhibitors with different potency show differential effects on the kinetics of DNA strand break rejoining.

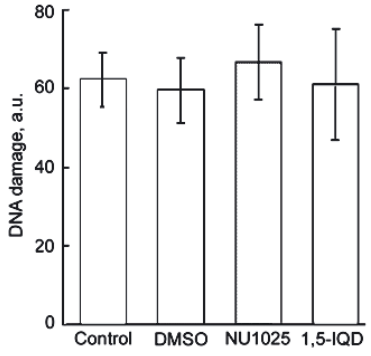

Figure 3. DNA damage in Raji cells incubated for 180 min in the presence of a PARP inhibitors alone (100 μM).

DNA damage in cells after exposure to H_2O_2 was reduced significantly by addition of DMSO (0.2%) alone, suggesting that caution should be used when using DMSO as a solvent for PARP inhibitors since it could mask the effect of an inhibitor in suppressing DNA repair. DMSO is a free-radical scavenger and can suppress the induction of DNA damage including the formation of micronuclei in X-irradiated CHO cells (Kashino *et al.*, 2007) and of strand-breaks in plasmids exposed to cadmium where it showed high level protection similar to that by catalase (Badisa *et al.*, 2007), and the background level of DNA damage was lower after incubation of lymphocytes with 1% DMSO for 10–30 min (Szyfter *et al.*, 2004). On the other hand, the activity of PARP itself was reduced nearly 20% in 4% DMSO (Banasik *et al.*, 2004).

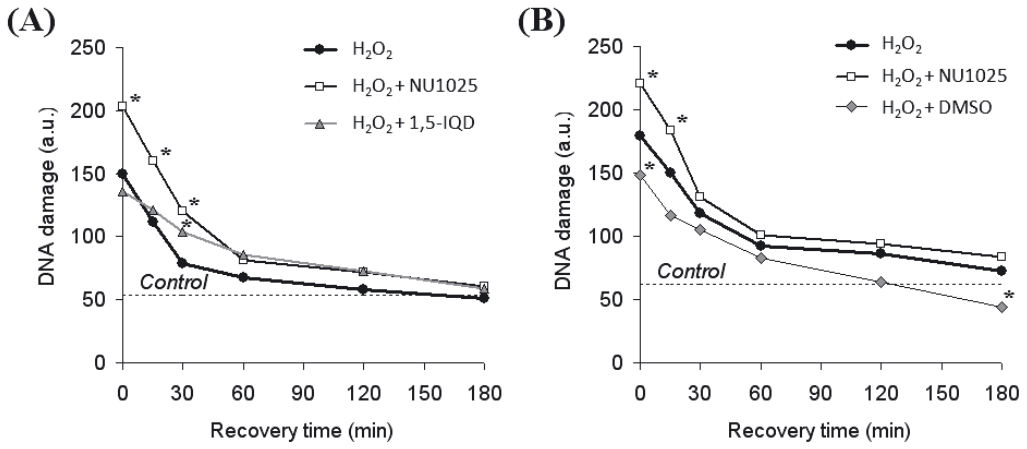

Figure 4. Effect of (A) NU1025 and 1,5-IQD; (B) NU1025 and DMSO alone on the kinetics of DNA strand break rejoining in Raji cells after exposure to H_2O_2.
The horizontal dashed lines show the level of strand breaks in control untreated cells, asterisks mark points significantly different from treatment with H_2O_2 alone.

PARP formed large foci in nuclei of cells exposed to H_2O_2, and during further incubation with NU1025 a significantly greater fraction of cells contained a large number of foci which persisted for more than one hour, corroborating results recently reported by Godon *et al.* (2008) who demonstrated that inhibition of PARP activity leads to accumulation of this enzyme in large foci at the DNA damage sites. In many intact cells accumulation of PARP was observed in nucleoli and exposure to DNA damaging agents caused its dispersion in the nucleoplasm (Desnoyers *et al.* 1996; Godon *et al.*, 2008; Rancourt & Satoh, 2009), but this phenomenon was not observed in our experiments with K562 cells. The difference may result from the specificity of highly proliferating K562 cells or from the difference in methods used during cell treatment with genotoxic agents or fixation. For example, Amé *et al.* (2008) have shown that accumulation of PARP in nucleoli may not be observed when 2% formaldehyde is used for cell fixation.

Here we present the results obtained for Raji and K562 cells but inhibition or stimulation of PARP activity in the first minutes after DNA damage also influenced the kinetics and efficiency of DNA repair in the next few hours in human lymphocytes and HL60 cells (Ryabokon, 2005; unpublished results). This influence could probably be exerted by changes in the order of protein recruitment to damaged sites (discussed in Ryabokon *et al.*, 2008), which could also affect the formation of stable PARP foci. Further research will be required to elucidate if PARP foci correspond to regions containing damaged DNA, their composition, and if they play a role in the recruitment of members of the BER pathway.

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