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Hydroxyurea-induced replication stress causes poly(ADP-ribose) polymerase-2 accumulation and changes its intranuclear location in root meristems of *Vicia faba*

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ABSTRACT

Replication stress induced by 24 and 48 h exposure to 2.5 mM hydroxyurea (HU) increased the activity of poly(ADP-ribose) polymerase-2 (PARP-2; EC 2.4.2.30) in root meristem cells of *Vicia faba*. An increase in the number of PARP-2 foci was accompanied by their delocalization from peripheral areas to the interior of the nucleus. Our results indicate that the increase in PARP-2 was connected with an increase in S139-phosphorylated H2AX histones. The findings suggest the possible role of PARP-2 in replication stress. We also confirm that the intranuclear location of PARP-2 depends on the duration of HU-induced replication stress, confirming the role of PARP-2 as an indicator of stress intensity. Finally, we conclude that the more intense the HU-mediated replication stress, the greater the probability of PARP-2 activation or H2AXS139 phosphorylation, but also the greater the chance of increasing the efficiency of repair processes and a return to normal cell cycle progression.

1. Introduction

Poly(ADP-ribose) polymerases (PARPs) are enzymes responsible for poly(ADP-ribosyl)ation, i.e. the covalent attachment of ADP-ribose subunits from NAD^+ (nicotinamide adenine dinucleotide) to target proteins involved in chromatin architecture and DNA metabolism. In mammals, the PARP superfamily consists of 18 enzymes. PARP-1 is the best known, but recent reports also emphasize the significance of other PARPs, i.e. PARP-2, centrosomal PARP-3, the vault-particle associated PARP-4 and even the telomeric and Golgi Tankyrase-1 and -2 (Amé et al., 2004). Tankyrase 1 (also known as PARP-5A) is a telomere-associated polymerase that is recruited to telomeres via interaction with telomeric-repeat binding factor 1 (TRF1) (Dregalla et al., 2010). Tankyrase 2 shares functions with Tankyrase 1 but is localized at several subcellular sites (the Golgi complex, nucleus, nuclear pore complexes, mitotic centrosomes and telomeres (Smith and de Lange, 1999)). PARP-1 and PARP-2 are polymerases that often share the same functions, and can heterodimerize and heteromodify each other *in vitro* (reviewed by Meder et al., 2004). However, there is evidence, based on a mouse knockout model, that PARP-2 cannot completely compensate for the loss of PARP-1 (Shieh et al., 1998; Amé et al., 1999). PARP activity has found to be enhanced in the vicinity of replication forks and in newly replicated DNA (reviewed by Bryant et al., 2009). Moreover, PARP-1 has been found to accumulate in transcriptionally active nucleoli (Meder et al., 2005). In plants, PARPs were first described 15 years ago. Six of nine PARP-like proteins identified so far in *Arabidopsis thaliana* do not have a catalytic domain, while three have catalytic activity (PARP-1-3) and are key factors involved in stress responses in plants (reviewed by Schulz et al., 2012). Additionally, a post-translational modification mediated by PARPs, *i.e.* poly(ADP-ribosyl)ation, plays an essential role in DNA damage/DNA repair, chromatin modification, control of transcription and cell death (Gibson and Kraus, 2012; Luo and Kraus, 2012; Burkle and Virag, 2013; Song et al., 2015).

Involvement of PARP proteins in the DNA repair process consists in binding of a dimeric PARP protein to damaged DNA (via zinc-finger domains located at the N-terminus of PARP), leading to

Abbreviations: DDR, DNA Damage Response; dNTP, deoxyribonucleoside triphosphates; DSBs, double-Strand Breaks; TEM, transmission electron microscopy; HU, hydroxyurea; NAD^+, nicotinamide adenine dinucleotide; NE, nuclear envelope; NER, nucleotide excision repair; pADPRT, poly(ADP-ribosyl) transferase; PAR, poly(ADP-ribose); PARG, poly(ADP-ribose) glycohydrolase; PARPs, poly(ADP-ribose) polymerases; PARP-2, poly(APD-ribose) polymerase-2; PCC, premature chromosome condensation; POLR2A, polymerase (RNA) II (DNA directed) polypeptide A; RNR, ribonucleotide reductase; SSBs, single-strand breaks; Tdp 1, tyrosyl DNA phosphodiesterase 1; TRF1, telomeric repeat binding factor 1; XRCC1, X-ray repair cross-complementing 1.

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the activation of the catalytic domain located at the C-terminus of PARP. Active PARP protein catalyzes the breakdown of NAD^+ into nicotinamide and ADP-ribose, and then joins ADP-ribose molecules to protein acceptors in the form of linear or branched chains of poly ADP-ribose (PAR) (Mendoza-Alvarez and Alvarez-Gonzales, 1993; Panzeter and Atthaus, 1994). PAR polymers are formed within seconds of detecting DNA damage, but as soon as after a few minutes are degraded by poly(ADP)ribose glycohydrolase (PARG) (Davidovic et al., 2001; Altmeyer et al., 2009). Robu et al. (2013) indicated that activated PARP-1 forms PAR polymers that post-translationally modify DNA repair-related proteins participating, for example, in the versatile nucleotide excision repair pathway (NER) that removes the DNA damage induced by UV. In turn, Jia et al. (2013) showed that PARPs are involved in non-homologous end joining repair (NHEJ) in *Arabidopsis*. In fact, the importance of the PAR polymer has been established in many cellular processes: (i) epigenetic regulation of chromatin dynamics (Poirier et al., 1982; Klenova and Ohlsson, 2005; Meyer-Ficca et al., 2005; Cohen-Armon et al., 2007; El Ramy et al., 2009; Quenet et al., 2009; Meyer-Ficca et al., 2013), (ii) detection, signaling and repair of DNA damage (Lankenau et al., 1999; Meder et al., 2004; De Vos et al., 2012; Tallis et al., 2014), (iii) genome integrity and mitotic progression (Boehler et al., 2011), (iv) genotoxic stress tolerance, programmed cell death, transcription and cell cycle control (Meder et al., 2004; Briggs and Bent, 2011; Lamb et al., 2012; reviewed by D'Amours et al., 1999), (v) regulation of intracellular transport processes (Abd Elmageed et al., 2012), as well as (vi) homeostasis of telomere length and sister telomere association (Dantzer et al., 2004; O'Connor et al., 2004; Gomez et al., 2006; Beneke et al., 2008). Some of these functions open stimulating prospects for targeting PARPs in cancer therapy (Boehler et al., 2011; De Vos et al., 2012). However, in most of the previous reports, conclusions about the functions of various PARPs have been ambiguous, as the effects of their mutations were almost always pleiotropic in nature.

Hydroxyurea (HU) is the best known and most commonly used inducer of replication stress in plant, animal and human cells. HU inhibits the replication fork in an indirect way, by blocking the catalytic domain of ribonucleotide reductase (RNR) (Koç et al., 2004; Alvino et al., 2007; Roa et al., 2009). The disappearance of RNR activity, converting ribonucleotides into deoxyribonucleoside triphosphates (dNTP), leads to: (i) a disruption in the quantitative balance of the precursors necessary for DNA synthesis (Chabes and Thelander, 2000; Osborn et al., 2002), and (ii) reduction in the rate of replication through the activation of S phase checkpoints (Hartsuiker et al., 2001; Bartek et al., 2004; Rybaczek and Kowalewicz-Kulbat, 2011; Rybaczek, 2014). Research on HU-induced S phase checkpoints has shown that, in spite of the inhibition of dNTP synthesis, the DNA replication process is not halted completely, but is only strongly limited in order to enable the stabilization of replication forks (Boddy and Russell, 2001; reviewed by Bartek et al., 2004) and to preserve the ability to continue DNA synthesis immediately after the removal of replication blocks, without the need of the assembly of replication complexes (Desany et al., 1998; Santocanale and Diffley, 1998; Shirahige et al., 1998; Dimitrova and Gilbert, 2000; Boddy and Russell, 2001). Depending on the type of cell, the inhibitor concentration and the incubation period, HU blocks cells at the G1/S transition (Sree et al., 2012; Winnicki et al., 2013), in the S phase (Hartsuiker et al., 2001; Winnicki and Maszewski, 2012), at the S/G2 transition (Rybaczek et al., 2008; Rybaczek, 2014) or in the G2 phase. HU changes the overall metabolism by transcription activation, and an increase in RNA polymerase II large subunit (POLR2A) content (Winnicki et al., 2013).

Ultrastructural studies on chromatin in HU-treated rad53 mutants revealed two classes of DNA structure not found in normal cells. The first consisted of long single-stranded fragments, while the second of cross-like structures known as Holliday junctions (Sogo et al., 2002). The presence of single-stranded fragments is an effect of the uncoordinated replication of both DNA strands (leading and delayed). In addition, at the site of DNA damage or in replication forks blocked by a replication inhibitor, Y-shaped replication forks may be subject to reversion and form Holliday junctions. When the repair of damaged DNA fragments by cutting and re-synthesis is not possible, the solution to the problem lies – out of necessity – in homologous recombination. PARP-1, by interacting with topoisomerase I (Top I), directs a DNA III ligase complex and factor X-ray repair cross-complementing 1 (XRCC1) to the sites of DNA damage, thereby preventing undesirable recombinant activities. PARP-1 binds initially to the Top I-DNA complex, and then becomes an acceptor for DNA III ligase complex with XRCC1 and tyrosyl DNA phosphodiesterase 1 (Tdp1) (Leppard et al., 2003; Okano et al., 2003; Leppard and Champoux, 2005).

The PARP superfamily has been well studied in animals, but there are only few reports on PARPs in plant-specific processes (e.g. Doucet-Chabeaud et al., 2001; Jia et al., 2013; Song et al., 2015). The molecular basis for PARP interaction in plants remains under-investigated.

The objective of this work was to establish the influence of HU on the amount of PARP-2, and the intranuclear localization of PARP-2 in root meristem cells of *V. faba*. Our experiments were designed to facilitate investigation of the relationship either between the number of PARP-2-specific foci and double-strand break (DSB) induction, or the number of PARP-2-specific foci and their intranuclear locations. Next, the behavior of PARP-2 foci (accumulation and re-distribution) and its DNA-repair function (in co-localization with H2AX histones phosphorylated on S139) were analyzed in order to characterize the role of PARP-2 in replication stress.

2. Material and methods

2.1. Plant material and growth conditions

Seeds of *Vicia faba* var. *minor* cv. Nadwiślański (Center for Seed Production in Sobiejuchy, Poland) were dark-germinated at room temperature on wet filter paper in Petri dishes. Four days after imbibition, 3 cm seedlings were selected and incubated in water, or 2.5 mM HU for 24 h, or 2.5 mM HU for 48 h, as described by Rybaczek et al. (2007). During germination and incubation, the roots were oriented horizontally and aerated continuously by gentle rotation of fluids in a water-bath shaker (30 rpm).

2.2. Antibodies

Immunocytochemical and biochemical detection of PARP-2 was performed using rabbit polyclonal antibodies from Agrisera (Vännas, Sweden; #AS10 675). The rabbit monoclonal antibodies specific to phospho-H2AX (Ser139) (20E3) were from Cell Signaling (Danvers, MA, USA; #9718). Bound primary antibodies in all investigated cases were detected with the secondary goat anti-rabbit IgG DyLight®488 antibody (Agrisera, Vännas, Sweden; #AS09 633, used for immunocytochemistry) and with the secondary anti-rabbit IgG (AP-linked) antibody (Cell Signaling, Danvers, MA, USA; #7054, used for immunoblotting). In the co-localization related experiments (PARP-2 versus phospho-H2AXS139), the anti-phospho-histone H2AX (Ser139) monoclonal antibody (clone JBW301) was used (Upstate/Millipore, Billerica, MA, USA; 05-636). Bound primary antibody, in this particular case, was detected with the secondary anti-mouse IgG AlexaFluor®594 conjugate antibody (Cell Signaling, Danvers, MA, USA; #8890). The mouse monoclonal antibody to β-actin (#A5441) and the sec-