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Effect of aging and oxidative/genotoxic stress on poly(ADP-ribose) polymerase-1 activity in rat brain

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Poly(ADP-ribose) polymerase-1 (PARP-1, EC 2.4.2.30), a DNA-bound enzyme, plays a key role in genome stability, but after overactivation can also be responsible for cell death. The aim of the present study was to investigate PARP-1 activity in the hippocampus, brain cortex, striatum and cerebellum in adult (4 months) and aged (24 months) specific pathogen free Wistar rats and to correlate it with PARP-1 protein level and p53 expression. Moreover, the response of PARP-1 in adult and aged hippocampus to oxidative/genotoxic stress was evaluated. Our data indicated a statistically significant enhancement of PARP-1 activity in aged hippocampus and cerebral cortex comparing to adults without statistically significant changes in PARP-1 protein level. The expression of p53 mRNA was elevated in all aged brain parts with the exception of the cerebral cortex. Our data suggest that enhancement of PARP-1 activity and p53 expression in aged brain may indicate higher DNA damage. Our data also indicate that during excessive oxidative/genotoxic stress there is no response of PARP-1 activity in aged hippocampus in contrast to a significant enhancement of PARP-1 activity in adults which may have important consequences for the physiology and pathology of the brain.

Keywords: PARP-1, poly(ADP-ribosyl)ation, brain, aging, oxidative stress, p53 protein, genotoxic stress

Poly(ADP-ribose) polymerase-1 (PARP-1, EC 2.4.2.30) is a highly conserved protein mainly localized in the nucleus. This about 113 kDa protein contains nuclear localization signal and DNA binding domain that recognizes DNA strand breaks. PARP-1 is activated by single and double DNA strand breaks, being the earliest and the most sensitive sensor of DNA damage (Malanga & Althaus, 2005). PARP-1 is responsible for the poly(ADP-ribosyl)ation of more than 40 nuclear chromatin-associated proteins, among them are p53, NF-κB, histones, AP-1, AP-2, topoisomerase I and II, and PARP itself. Automodified PARP-1 attracts the base excision repair complex facilitating removal of DNA damage (Masson *et al.*, 1998; Pleschke *et al.*, 2000; Strosznajder *et al.*, 2005). A lack of PARP-1 activity causes hypersensitivity to genotoxic agents and elevates chromosomal abnormalities. Overactivation of PARP-1 by massive DNA breakage (Zhang *et al.*, 1994) leads to a depletion of its substrate βNAD$^+$ and of ATP. These events are responsible for alteration of mitochondrial potential and may be involved in the release of apoptosis inducing factor (AIF) (Yu *et al.*, 2002). Moreover, PARP-1 interacts with NF-κB and other transcription factors (Hassa & Hottiger, 1999; Oliver *et al.*, 1999; Wesierska-Gadek & Schmid, 2001; Chiarugi & Moskovitz, 2003; Wesierska-Gadek *et al.*, 2003; 2005). A growing body of evidence indicates the importance of PARP-1 in cell death during ischemia and neurodegenerative diseases (Strosznajder *et al.*, 2003; 2005). The role of PARP-1 as a "guardian of the genome" has raised question about its significance in aging. Numerous publications have documented increased levels of damaged DNA and mutation frequency in aged organs (Mandavilli & Rao, 1996; Izzotti *et al.*, 1999; Hamilton *et al.*, 2001; Cabelof *et al.*, 2002), thus it was of great interest to elucidate the influence of aging on PARP

Abbreviations: 3-AB, 3-aminobezamide; AIF, apoptosis-inducing factor; AP-1 and -2, activator protein-1 and –2; BSA, bovine serum albumin; DTT, DL-dithiothreitol; ECL, enhanced chemiluminescence; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IR, immunoreactivity; MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; βNAD$^+$, beta-nicotinamide adenine dinucleotide; NF-κB, nuclear factor kappa B; NMDA, *N*-methyl-D-aspartate; P1, crude nuclear fraction; PMSF, phenylmethylsulfonyl fluoride; PARP-1, poly(ADP-ribose) polymerase-1; PBS-T, phosphate-buffered saline with 0.05% Tween-20; ROS, reactive oxygen species; RT-PCR, reverse transcription and polymerase chain reaction; SPF, specific pathogen free.

expression and activity. Increased PARP activity during aging has been reported previously (Pero *et al.*, 1985; Bizec *et al.*, 1989; Grube & Bürkle, 1992; Messripour *et al.*, 1994; Muiras *et al.*, 1998) but the PARP activity and its expression at the protein level in mammalian aged brain has not been fully elucidated. Moreover, the relationship between PARP-1 and p53 in aged brain is not well understood. In our previous work we described changes of poly(ADP-ribosyl)ation that occurred with age in brains of non specific pathogen free (NSPF) rats (Strosznajder *et al.*, 2000a; 2000b). The activity of PARP-1 was elevated in aged brain cortex and cerebellum but it was significantly lower in aged hippocampus comparing to adult (Strosznajder *et al.*, 2000a). The aim of the present study was to investigate the PARP-1 activity in correlation to PARP-1 protein level and expression of p53 in aged brain parts comparing to adults from the specific pathogen free (SPF) health category animals. Moreover, the response of PARP-1 activity to oxidative/genotoxic stress in adult and aged hippocampus was examined.

MATERIALS AND METHODS

Male Wistar adult (4 months) and old rats (24–27 months) were obtained from the Animal Breeding House of the Medical Research Centre, Polish Academy of Sciences (Warszawa, Poland). The animals were kept behind a barrier system and were of the specific pathogen free health category. The Local Medical Research Centre Ethics Committee that followed the European Communities Council Directive of 24 November 1986 accepted the use of these animals for the described experiments. [Adenine-^{14}C]nicotinamide adenine dinucleotide (NAD) was from Amersham (Buckinghamshire, England). Protease Inhibitor Cocktail Complete was obtained from Boehringer Mannheim (Germany). β-Nicotinamide adenine dinucleotide (β-NAD), DL-dithiothreitol (DTT), 3-aminobezamide (3-AB), and other reagents were obtained from Sigma (St. Louis, MO, USA).

The animals were quickly decapitated. Brain parts were isolated on ice. A 10% homogenate (w/v) was immediately prepared in a Dounce-type glass homogeniser in ice-cold 0.25 M sucrose with 50 mM NaCl, 1 mM EDTA and 1 mM dithiothreitol in 10 mM Tris/HCl, pH 7.4, with "Complete™" protease inhibitors. The homogenate was centrifuged for 4 min at 600 × *g* to obtain crude nuclear fraction (P1) pellet which was resuspended in half volume of the homogenisation buffer and used for PARP-1 protein and activity measurements.

Determination of basal PARP-1 activity in adult and aged brain parts. PARP-1 activity was determined using [adenine-^{14}C]NAD as a substrate. The incubation mixture in a final volume of 100 μl contained 200 μM βNAD and 4 × 10^5 d.p.m. [adenine-^{14}C]NAD, 100 mM Tris/HCl buffer (pH 8.0), 10 mM $MgCl_2$, 5 mM DTT, 50 μM PMSF and 200 μg of protein. The mixture was incubated for 1 min at 37°C and the reaction was stopped by adding 0.8 ml of ice-cold 25% trichloroacetic acid. Precipitates were collected on Whatman GF/B filters, washed three times with 5% trichloroacetic acid and left overnight for drying. The radioactivity was measured using an LKB, Wallach 1409 scintillator counter.

Determination of basal PARP-1 protein level in adult and aged brain parts. PARP-1 protein immunoreactivity was measured by Western-blot. The P1 nuclear fraction was denatured for 5 min at 95ºC and electrophoresed through 7.5% SDS/polyacrylamide gel. The proteins were transferred onto Hybond™ C-Extra nitrocellulose membrane (Amersham). The membrane was blocked in 1% bovine serum albumin (BSA) in PBS-T for 1 h at room temperature and probed with 1:500 monoclonal anti-PARP-1 antibody (Sigma, clone# C-2-10) in 0.5% BSA overnight at 4ºC and then with 1:2500 secondary horseradish peroxidase-linked anti-rabbit IgG (Amersham) in 5% non-fat dry milk for 1 h at room temp. The bands were visualized with ECL kit (Amersham) and analyzed with a densitometer.

The same blots were then stripped for 30 min at room temp. in 50 mM glycine/HCl, pH 2.0, with 1% SDS, blocked for 1 h at room temp. in 5% non fat dry milk in PBS-T and probed for 2 h at room temp. with 1/400 C4 anti-actin antibody in 0.1% BSA. After probing with 1/5000 anti-mouse secondary antibody in 5% milk for 1 h at room temp. the intensities of actin bands were recorded. The results were expressed as the ratio of densities of PARP-1 bands to actin bands in the same lanes.

Determination of p53 mRNA level in adult and aged rat brain parts. Total RNA was extracted from brain cortex, hippocampus, striatum and cerebellum from adult and aged rats using TRI REAGENT isolation kit (Sigma, St. Louis, MO, USA) according to the manufacturer's procedure. The yield and quality of RNA were determined by measuring the absorbance at 260 and 280 nm in a spectrophotometer. First-strand cDNA was synthesized from 5 μg of total RNA by reverse transcription (RT), using Reverse Transcription System (Promega Corporation, Madison, WI, USA). The reaction was performed in a final volume of 20 μl using 1500 units of AMV reverse transcriptase, 0.5 μg oligo(dT) primer, 2500 units of RNase inhibitor, 1 mM dNTP mix, 5 mM $MgCl_2$ and RT buffer in one cycle: 42°C for 1 h and 99°C for 5 min with subsequent cooling to 4°C.

Polymerase chain reaction (PCR) was carried out using *Taq* PCR Master Mix Kit (Qiagen, GmbH, Germany) according to the manufacturer's pro-

cedure in a total volume of 50 μl with 20 pmol of each primer. The primer sequences for p53 were 5′-TTCCCTCAATAAGCTGTTCTGCC-3′ (forward) and 5′-TGCTCTCTTTGCACTCCCTGG-3′ (reverse). As an internal control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was coamplified using primers 5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′ (forward) and 5′-CATGTGGGCCATGAGGTCCACCAC-3′ (reverse). Each PCR cycle consisted of denaturation at 94°C for 1 min, annealing at 66°C for 1 min, and extension at 72°C for 1 min, followed by a final 7-min extension at 72°C. PCR amplification was carried out for 36 cycles using a Perkin-Elmer GeneAmp 2400 thermal cycler. After amplification, samples were separated on 2% agarose gel containing 200 μg/l ethidium bromide in 0.5× Tris/borate/EDTA buffer. The intensity of the p53 and GAPDH bands was estimated by densitometric analysis of the gel in UV light using a NucleoVision apparatus and GelExpert 4.0 software from NucleoTech.

Determination of PARP-1 activity in adult and aged hippocampus subjected to oxidative and genotoxic stress. For the determination of free radical-stimulated PARP-1 activity, the P1 nuclear fraction from hippocampus was preincubated with 25 μM $FeCl_2$ and 10 μM ascorbate for 1.5, 15 and 60 min. Then [adenine-^{14}C]NAD was added and the reaction was carried as was described above. For determination of the effect of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG), the P1 nuclear fraction from hippocampus was pre-incubated for 15 min at 37°C and then incubated with 50 μM MNNG for 5 min. Then 200 μM βNAD$^+$ was added and the reaction was carried out for 1 min at 37°C. The reaction was terminated with denaturing sample buffer and the material was subjected to electrophoresis and transfer as described above. The membrane was blocked overnight in 5% non-fat dry milk and probed with 1:400 anti-poly(ADP-ribose) antibody (Alexis, clone# 10H) in 0.25% milk and then with 1:3000 secondary horseradish peroxidase-linked anti-rabbit IgG (Amersham) in milk. The bands were visualized with the ECL kit (Amersham) and analysed with a densitometer.

RESULTS AND DISCUSSION

Our present results demonstrate that in SPF animals PARP-1 activity is significantly elevated by 52% in aged hippocampus comparing to the adult one (Fig. 1). The activity of this enzyme in the cerebral cortex was also significantly enhanced by 64% while in cerebellum and striatum it was non-significantly elevated by 24% and 16%, respectively (Fig. 1). Our previous experiments demonstrated a significantly lower PARP-1 activity in aged *versus* adult hippocampus from non-SPF-category animals (Strosznajder *et al.*, 2000a). We suggest that PARP-1 in non-SPF animals was subjected to excessive stress evoked by some kind of infection which markedly affected this enzyme in the hippocampus, leading in consequence to its covalent modification and inhibition. In the cerebral cortex and cerebellum the values were very similar to those observed previously (Strosznajder *et al.*, 2000a). Moreover, in the current study we also estimated the PARP-1 protein level. The immunoreactivity was unchanged in the aged hippocampus and cerebellum, non-significantly lowered in aged cerebral cortex and significantly down-regulated in the striatum of old rats (Fig. 2). These results indicate that the age related changes of PARP-1 occurr at the level of enzymatic activity, not on the protein expression level. Also Grube and Bürkle (1992) presented a lack of correlation between the amount of PARP protein and the life span with strong positive correlation between PARP activity and life span. They observed a higher specific enzyme activity in longer-lived species. In contrast, Messripour *et al.* (1994) demonstrated an enhancement of PARP activity and also PARP protein level in neurons and glia from aged rat brain. It is known from previous studies that PARP regulates/modulates p53 and NF-κB function (Malanga *et al.*, 1998; Chiarugi, 2002; Wesierska-Gadek *et al.*, 2003). It has been demonstrated that the activity of PARP-1 regulates expression of p53 (Agarwal *et al.*, 1997; Wang *et al.*, 1998). Our data indicated no changes of p53 expression in aged brain cortex comparing to the adult one and a non-significant enhancement of p53 mRNA level in the hippocampus and striatum (Fig. 3). However, significantly elevated p53 mRNA expression was found in aged cerebellum (Fig. 3). These results correlate with the higher PARP-1 activity in aged brain parts with the exception of brain cortex where p53 mRNA was not elevated. The data of Chung *et al.* (2000) presented higher immunoreactivity (IR) of p53 in the hippocampus and cerebel-

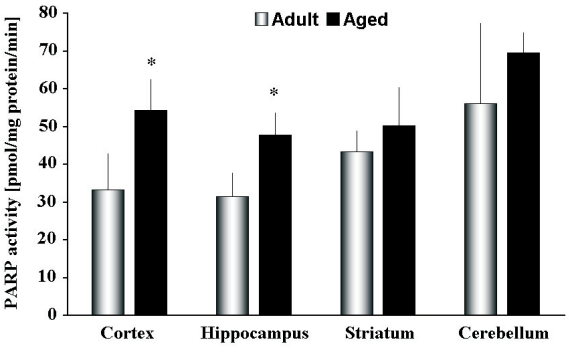

Figure 1. PARP-1 activity in nuclear fraction from different parts of adult and aged brains.
Bars represent values (means ±S.E.M.) from 3–5 animals. *$P < 0.05$ comparing to values from corresponding adult brain parts. Student's *t*-test.

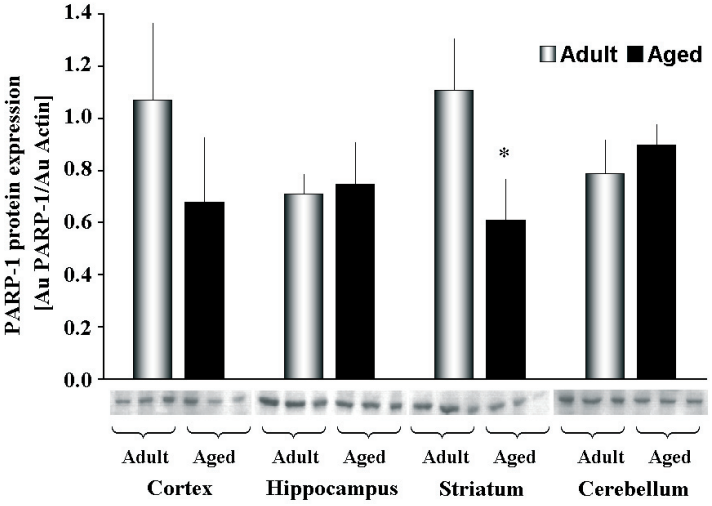

Figure 2. PARP-1 protein level in nuclear fraction from different parts of adult and aged brains.
Bar represent values (means ±S.E.M.) from 3 animals. *$P < 0.05$ comparing to values from corresponding adult brain parts. Student's *t*-test.

lum of aged rats. They did not observed p53 IR in the brain of adult rats. The increased expression of p53 seen with DNA damage indicated a possible link between senescence and DNA damage. To better understand the aged-related changes of PARP-1 we also determinated in *in vitro* experiments the response of PARP-1 from adult and aged hippocampus to oxidative and genotoxic stress. In contrast to our previous approach when we stimulated free radical generation by ligating glutamatergic NMDA receptors or by adding extracellular β-amyloid (Strosznajder *et al.*, 2000b), our current study was more direct, independent of age-related changes in signaling pathways, expression of proteins, and other factors that might uncontrollably change with age. $FeCl_2$ (25 μM) and ascorbate (10 μM) which generated the oxidative stress (1.5 and 15 min) stimulated significantly the PARP-1 activity in the adult tissue (Table 1, Fig. 4). In aged hippocampus PARP-1 activity was near the control value (Table 1, Fig. 4). However, a prolonged, 60 min oxidative stress had an inhibitory effect on PARP-1 activity in aged and also in adult hippocampus. The enzyme activity was about 40% of the corresponding control value (Fig. 4). Damage of DNA by alkylating agent MNNG also caused strong, significant increase of poly(ADP-ribosyl)ation in the adult (258% of control) but not in aged hippocampus (Table 1). Reduced PARP activation in response to enzymatic DNA cleavage was reported by Malanga *et al.* (2005) in aged rat cerebellum. Our

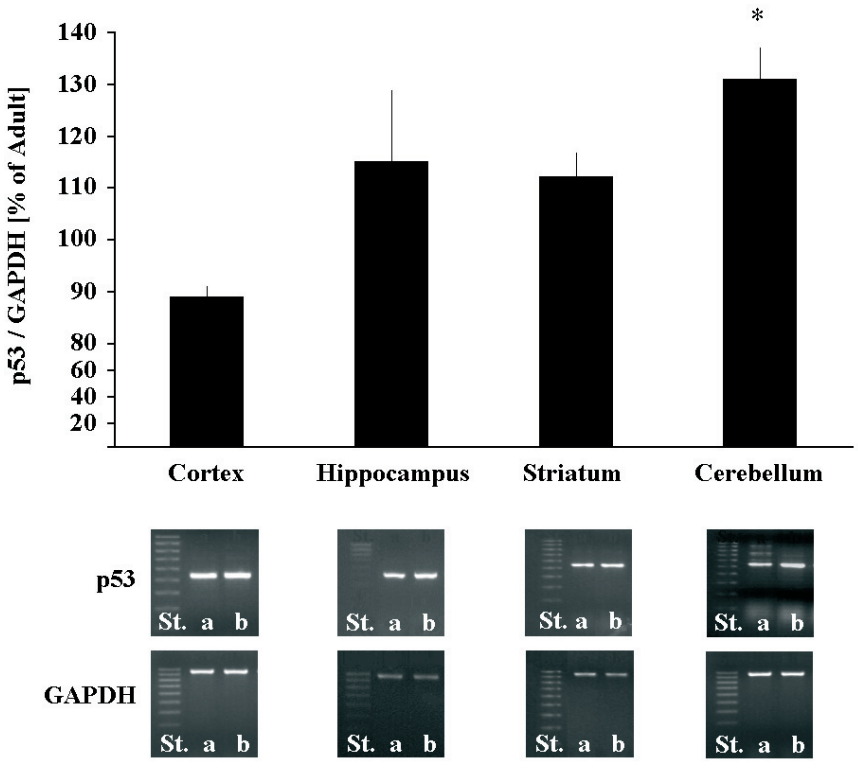

Figure 3. p53 mRNA expression in adult and aged rat brain.
The mRNA ratio was calculated by dividing p53 mRNA levels by the corresponding GAPDH mRNA levels measured in the same experiment. Bars represent values (means ±S.E.M.) from 8 animals. Values from adult brains were taken as 100%. Differences between adult (a) and aged (b) animals were analyzed using unpaired Student's *t*-test, *$P < 0.05$.

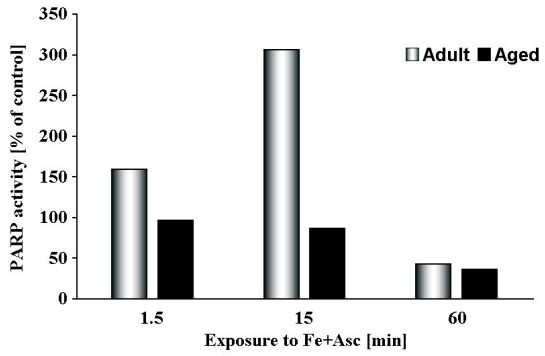

Figure 4. Effect of *in vitro* oxidative stress on PARP-1 activity in nuclear fraction from adult and aged hippocampus.
Nuclear fractions from adult and aged hippocampus were preincubated in the presence of 25 μM $FeCl_2$ and 10 μM ascorbic acid for 1.5, 15 and 60 min. Control values from non-treated adult and aged hippocampus were taken in each case as 100%. The values are means from one typical experiment carried out in triplicate. Pooled material from 3 adult and 2 aged animals was used.

data indicate that PARP-1 in aged hippocampus has a lower ability to respond to excessive oxidative or genotoxic stress. In consequence, the alteration of PARP-1 may have a protective effect against energy depletion but may also lead to a decreased efficiency of DNA repair machinery.

Table 1. PARP activity in adult and aged hippocampus subjected to oxidative and genotoxic stress

	% of control	
Conditions	Adult	Aged
25 μM $FeCl_2$ + 10 μM Asc	157 ± 22*	111 ± 22
50 μM MNNG	258 ± 46*	83 ± 12

P1 nuclear fraction from hippocampus was incubated with $FeCl_2$ and ascorbate (Asc) for 1.5 min. Then enzymatic activity was measured using [adenine-^{14}C]NAD as described in the text. P1 nuclear fraction from hippocampus was incubated for 5 min with MNNG and subjected to Western blot for poly(ADP-ribose) as described in the text. Control values from non-treated adult and aged hippocampus were taken in each case as 100%. The values are means of 3–4 experiments ±S.E.M. *$P < 0.05$, Student's *t*-test.

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