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# Alteration of ADP-ribosylation in aging rat brain astrocytes

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**Key words:** ADP-ribosyl polymerase, DNA damages, Glutamine synthetase

**Abstract:** DNA damage and the enzyme poly (ADP-ribose) polymerase (PARP) associated with the pathogenesis of numerous age-related neurodegenerative disorders. Astrocytes play crucial roles in both support metabolic functions and cell viability of the brain. PARP regulates DNA damage and repair in the brain cells. In this study PARP activity and DNA strand break were investigated in the astrocytes isolated from young and aged rat brain. Three and 30-month-old rats were killed by decapitation and brains were removed onto an ice cooled glass plate. Astrocytes were isolated by sucrose density gradient centrifugation and glutamine synthetase (GS) served as a marker of the astrocytes lineage. The specific activity of PARP was assayed in permeabilized cells by measuring the incorporation of the ADP-ribose moiety of [3H]NAD into the nuclear acceptor proteins. The rate of DNA strand breaks was determined using a fluorescent dye and monitored spectrofluorimetry. An increase (about 75%) in the PARP activity was observed in the whole homogenates of aged rats, whereas this rise was more pronounced (about 360%) when the reaction was measured in the purified astrocyte preparations. The amount of DNA strand breaks was also higher in the astrocytes isolated from the aged brain as compared to that of young levels. The close relationship between the level of DNA strand breaks and PARP activity in the astrocytes suggest that these cells are susceptible to the metabolic alterations in aging. It is concluded that the astrocytes PARP might be considered as a therapeutic target for combating age related neurodegenerative disorders.

## Introduction

Nuclear poly ADP-ribosylation mediates a post-translational reaction that modifies several protein structures. The reaction is catalyzed by poly ADP-ribose polymerase [PARP, Ee 2.4.2.30], which transfers ADP-ribose moiety of $NAD^+$ to nuclear acceptor proteins, leading to long chain polymers of ADP-ribose (Liu *et al.*, 2017). The main role of PARP is to detect and initiate an immediate cellular response to metabolic, chemical, or radiation-induced single-strand DNA breaks.

Previous studies have shown the accumulation of an increasing amount of single-stranded regions in the DNA with age activity (Mandel, 1990). It has been reported that agents that provoke DNA damage produce activation of PARP (Mandel, 1990). Overactive PARP depletes its substrate $NAD^+$, slowing the rate of glucose metabolism, electron transport, and ATP formation, eventually leading to functional impairment and cell death, as well as up-regulation of several pro-inflammatory pathways (Ying *et al.*, 2003). Convincing evidence has shown that deficient DNA repair allows greater naturally-occurring accumulation of DNA damages in aging (Liu and Yu, 2015; Lilyestrom *et al.*, 2010; Best, 2009). These observations support the view that PARP activation is closely connected to various pathophysiological conditions. Experimental studies have demonstrated that age-associated increases of DNA damage in the brain may be a challenging problem for reconstruction because neurons may be difficult to replace and DNA repair could be difficult (Best, 2009; Freitas and de Magalhães, 2011; Burhans and Weinberger, 2007). Therefore, in traumatic brain injury, PARP activation induces neuronal cell death as well as microglial activation, and related neurotoxicity contributes to subsequent neurological dysfunction (Stoica *et al.*, 2014; Boulu *et al.*, 2001).

Astrocyte-neuron interactions perform numerous functions; including, buffering the extracellular space, providing certain substrates to neurons, and regulating some neurotransmitters, brain energy metabolism and the overall brain homeostasis (Bambrick *et al.*, 2004; Schiweck *et al.*, 2018; Ying *et al.*, 2003; Faissner *et al.*, 2010; Nagethus *et al.*, 2013; Dienel, 2013). The results of these studies have led to the predominant view that the loss of astrocyte functions contributes to the aging brain and neurodegenerative diseases. Although the impact of ADP-ribosylation in aging is very well-documented,

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its effects on astrocytes have not been fully established. The aim of the current study was to assess ADP-ribosylation and DNA strand breaks in isolated astrocyte in aging rat.

## Material and Methods

*Animals*

Male albino rats of the Wistar strain were used; ad libitum feeding and drinking water were implicated. The ages of the animals were carefully recorded from birth. Young rats (3 months old) with weight ranging from 200 to 250 g and aged animals (30 months old) with weight ranging from 650 to 720 g were used. Animals were maintained according to the animal welfare regulation in animal house until the desired age was attained.

*Astrocyte isolation*

Astrocytes were isolated from rat brain according to the method of Farooq and Norton (1978). In each experiment, three rats were killed by decapitation and brains were removed onto an ice-cooled glass plate over crushed ice and chopped into the consistency of the mince, which was rapidly transferred into 30-35 ml of an ice cold solution of 7.5% (w/v) polyvinylpyrrolidone (PVP, average mol. wt. 40000, Sigma), 1% BSA and 10 mM $CaCl_2$. The minced tissue was eased into a disposable syringe, and the plunger was used to push the mince suspension through screens of 110 µm pore size nylon mesh. The cell suspension was then passed three times through 80 µm stainless steel screen using the disposable syringe. After 20 min on ice, 5 ml of the resulting crude cell suspension was layered over a two-step gradient consisting from the bottom-up of 5 ml 1.75 M sucrose and 6 ml of 1.0 M sucrose containing 1% (w/v) BSA, and centrifuged at 41000 *g* for 30 min. Purified astrocytes were obtained as a pellet at the 1.0-1.75 M sucrose interface. The cells were washed three times using 0.32 M sucrose solution. The morphological characteristics of the cell preparation were examined under phase contrast microscopy. The characteristic star-like cell shape was easily recognized (Farooq and Norton, 1978). Some broken astrocytes were also seen.

*Astrocyte identification*

The enzyme glutamine synthetase (GS), which is a specific enzyme marker of astrocytes (Norenberg and Martinez-Hernandez, 1979; Suárez *et al.*, 2002), was assayed by ELISA to identify the relative purity of the astrocytes compared with the brain homogenates. The frozen-thawed cell fractions or the homogenate were diluted to 25 µg protein/ml by addition of bicarbonate buffer (50 mM, pH 8.5). In each ELISA immune-plate well, 200 µl of the diluted sample was added and allowed to bind to the plates overnight, at 4°C. The following day the wells were washed three times with the buffer and then incubated with 1% BSA for 1 h to block excess protein binding sites. The wells were washed with 0.1% Tween 20 (Sigma) in the bicarbonate buffer. The primary antibody (rabbit anti-glutamine synthetase) was added at optimal dilution and allowed to stand at room temperature. After 3 h, the wells were washed three times with bicarbonate/Tween-20 solution and then incubated with a 1:500 dilution of rabbit anti-mouse IgG-peroxidase conjugated (Pierce) for 2 h at room temperature. After three final wash steps, the wells were incubated with 100 µl of the chromogenic reagent (50 mM 5-amino-salicylsaure and 5 mM $H_2O_2$ in a 0.1 M sodium acetate solution (pH 5.5) for 15 min. The reaction was stopped by addition of 100 µl of sodium hydroxide solution, and the absorbance was measured at 450 nm in a micro-ELISA reader. The absorbance was linear with the time for the first 20 min of the peroxidase-catalyzed reaction and also with the protein concentration of 100 µg protein/ml. Duplicate wells were prepared for each sample, and the mean absorbance was used in subsequent calculations. On each plate, sheep brain GS (Sigma) in a range concentration of 50-1000 ng/ml was used as standard. Protein concentration was performed by the method of Lowry *et al.* (1951) with bovine plasma albumin as the standard.

*Assay of ADP-ribosyl polymerase activity*

Endogenous PARP activity was assayed in permeabilized cells by measuring the incorporation of the ADP-ribose moiety of [$^3$H]$NAD^+$ into acceptor material as previously described (Berger *et al.*, 1979). The incubation mixture of 120 mM Tris/HCI (pH 7.8), 4 mM $MgCl_2$, 10 mM dithiothreitol, 3 mM 2-mercaptoethanol, 0.048% Triton-X100, and 10 nM of [adenine-$^3$H]$NAD^+$ (specific activity, 10, mCi/nmol). The reaction was started by adding 25 µl of permeabilized cells ($10^5$-$10^6$ cells) in a final volume of 125 µl and carried out for 10 min at 37°C. The reaction was terminated by cooling the samples in ice and adding 2 ml of 10% trichloroacetic acid (TCA) containing 0.02 M sodium pyrophosphate. After 30 min at 37°C, the precipitate was collected on a Whatman GF/B glass fiber paper, washed four times with 10 ml of ice cold 5% TCA containing 0.02 M sodium pyrophosphate and once with 5 ml of ethanol/ether (1:1 vol/vol) solution. After drying the paper, radioactivity was measured by liquid scintillation counting. One unit of the enzyme activity was defined as the amount of the enzyme that catalyzes the incorporation of 1 pmol of ADP-ribose from $NAD^+$ into an acid-insoluble form per mg DNA per min at 37°C.

*Determination of DNA strand breaks*

DNA strand breaks were measured using the method described by Kanter and Schwartz (1982). The cell lysates were exposed to alkaline solution and the rate of DNA strand breaks was determined directly using ethidium bromide solution (6.7 *µg*/ml, 13.3 mM NaOH) as a fluorescent dye. The fluorescence was monitored at excitation of 520 nm and emission of 590 nm. Total DNA in the astrocyte lysates was measured by the fluorometric assay of Downs and Wilfinger (1983). Briefly, DNA was extracted and utilized in conjunction with Hoechst (bisbenzimidazole) reagent to obtain quantitative estimates of DNA levels in the cell or tissue extracts using bovine DNA as standard.

*Statistical analysis*

Results were expressed as mean ± SD. The intergroup variations were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's test, using SPSS. Data are mean ± SD values of duplicate determinations from three independent experiments. Statistical level of significance was set at $^*P < 0.05$ and $^{**}P < 0.005$.

## Results

The data of this study can be analyzed from four different perspectives: (a) the relative purities of the astrocyte preparations; (b) the relative PARP activity of the isolated cells; (c) the relative rate of DNA strand breaks in the cells; and (d) any differences that are related to the age of the animals under study. The levels of GS of whole brain homogenates from young rats were not significantly different as compared to that of aged rats (Fig. 1). Although GS contents of astrocyte preparations were higher (about 36%), its levels were virtually similar in young and aged animals.

Cellular preparations were used to investigate PARP activity in the aging brain. As shown in Fig. 2, when PARP activity of the astrocytes was measured by incorporation of [3H]NAD to the acceptor molecules, a substantial increase in PARP activity was recorded in the cells prepared from the aged rat brain as compared to that of the young animals (> 3-fold). However, a relatively lower level of increase (about 75%) was observed in the enzyme activity in the brain homogenate of the aged rat brain (Fig. 2). The amount of DNA strand breaks was also determined in the homogenates and astrocytes with respect to age. As can be seen in Fig. 3, aging resulted in a marked increase in the amounts of DNA strand breaks in both cell samples in aging.

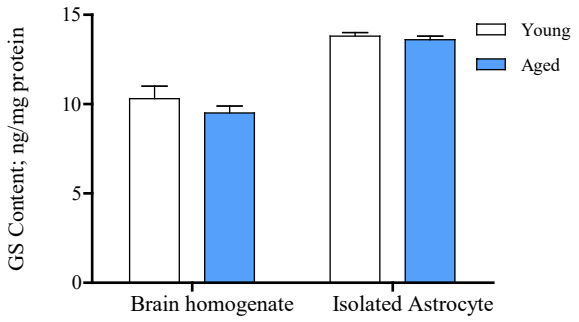


**FIGURE 1.** GS content of rat brain homogenate and isolated astrocytes. The concentrations of GS were measured in the lysed homogenate or isolated astrocytes from young and aged rat brain by ELISA. Data are mean ± SD values of duplicate determinations from three independent experiments. The differences between young and aged groups were not statistically significant.

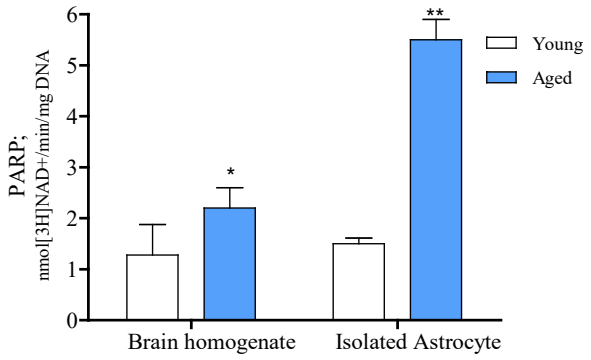


**FIGURE 2.** Effect of aging on PARP specific activity in rat brain homogenate and isolated astrocytes. The enzyme activity was assayed in permeabilized cells from young and aged rat brain by incorporation of [3H]NAD+ into acceptor materials. The specific activity of the enzyme was calculated on the bases of the amount of the [3H]$NAD^+$ incorporation/min/mg DNA. Data are mean ± SD values of duplicate determinations from three independent experiments. Statistical level of significance was set at $^*P < 0.05$ and $^{**}P < 0.005$.

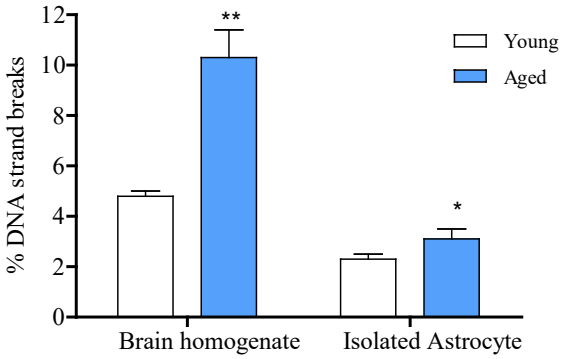


**FIGURE 3.** Effect of aging on DNA strand breaks in the rat brain homogenate and astrocytes. DNA in extracted cells from young and aged rat brain samples utilized directly in a sensitive fluorescence-enhancement assay with Hoechst reagent. The amount of DNA strand breaks were determined spectrofluorometry and presented as the percent of the total DNA contents. Data are mean ± SD values of duplicate determinations from three independent experiments. Statistical level of significance was set at $^*P < 0.05$ and $^{**}P < 0.005$.

## Discussion

This paper reports that the PARP specific activity of the aged rat astrocyte preparation is significantly higher than that of the young rat cell fraction. It is rather intriguing because most studies seem to agree that the activity of many enzymes decrease in the aging brain (Kitani, 2007). Although there may be some kind of artifact of cell damage during cell separations, in agreement with the previous report (Rose *et al.*, 2013), the relative purity of the cell fractions as judged by high GS activity was adequate to investigate the effect of aging on the cells. The results indicated that there is a close relationship between the rate of DNA strand breaks and ADP-ribosylation. Because the nuclear DNA and ADP-ribosylation of nuclear proteins are obviously implicated in the same nuclear processes (Mandel *et al.*, 1986), it seems that any changes in DNA integrity may lead to an alteration in the rate of ADP-ribosylation. Indeed, not only for this reaction, but many fundamental biological activities of these cells are tightly associated with the structural integrity of nuclear DNA (Liu *et al.*, 2013; Mandel, 1990). Therefore, the increase in the amount of DNA strand breaks in the aged rat brain astrocytes, as compared to young rat brain cells, may result from accumulation of this damage in the background of the functional DNA repair response. This indicates that cell death is increased in aged astrocytes compared with young ones as DNA strand breaks and PARP activity are highly correlated with cell death. However, the results of this study indicated that the lower occurrence of DNA strand breaks in young animals, under normal physiological conditions and adversative outcomes of PARP activity on the astrocyte, may be even beneficial due to rapid repair of DNA damages (Malanga and Althaus, 2005; Krishnakumar and Kraus, 2010). Conversely, at old age, the higher activity of the enzyme may

well be a cause of some of the well-documented phenotypes of aging, inflammation and eventually cell death. This suggestion is in agreement with the suggestion made by Ceccaldi *et al.* (2016) that repair pathway choice depends on a number of factors, including the type and severity of the damage, cell cycle stage, chromatin status and age of the organism.

In conclusion, these studies suggest that targeting PARP activity, which effectively attenuates astrocyte activation, may be considered as a therapeutic strategy to develop novel treatment options for the protection of astroglial cells, under conditions of brain injury or pathology.

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