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ALTERATIONS OF ADP-RIBOSYLATION AND DNA-BREAKS IN AGING BRAIN CELLS

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ABSTRACT

Neuronal and astroglial cells were prepared from whole brain of three month and 30-month- old rats for study of alterations in the nuclear poly ADP-ribosylation and DNA breaks with age. The relative purity of the cell preparations was confirmed by the determination of the neurofilament (low molecular weight) and glutamine synthetase content of the cells using ELISA. An increase (75%) in the poly ADP-ribosylation was observed in the whole brain cell suspension of aged rats, whereas the increase was markedly pronounced (460%) when the reaction was measured in the purified neuronal preparations. The rate of poly ADP-ribosylation in the astroglial fractions prepared from aged rat brain was higher than that of adult levels (67%). An unexpectedly high increase of ADP-ribosylation in the neurons and a much lower rate in the astroglial cells was thus recorded. The amount of DNA breaks was also higher in the neuronal preparation in aged brain as compared to that of adult levels. The amount of DNA breaks was much lower in the astroglial cells and aging had no effect on DNA breaks of these cells. The close relationship between DNA breaks and poly ADP-ribosylation in the different cell types suggest that neurons are more susceptible to the metabolic alterations of the aging process.

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INTRODUCTION

Numerous biochemical changes have been reported to occur in aging brain. A number of evidences are in favour of significant changes in the activities of various enzymes.[11,14,28,32,36,38] However, the molecular mechanism of the enzymatic alterations in the aged brain has not been well elucidated. Age- related changes in the metabolism of nucleic acids,[33] the DNA-damage and DNA- repair,[12,17] gene regulation,[21] and also alterations in the cellular content of ribosomes and RNA,[33] are thought to be important and could possibly lead to altered protein synthesis in aging brain. Moreover, the post- translational modifications of the proteins may be proposed either as the causative factors or as consequences of aging.

ADP- ribosylation is known as a post- translational reaction that modifies several proteins' structure.[3,34] The reaction is catalyzed by poly ADP- ribose polymerase [EC2.4.2.30] which transfers ADP- ribose moiety of NAD^+ to nuclear acceptor proteins, leading to long chain polymers of ADP- ribose. ADP- ribosylation seems to be involved in the regulation of DNA replication,[1,5] DNA repair,[2,9] cell proliferation,[6,13,27] cell differentiation,[7] and several other crucial biological functions (for review, see Mandel, 1990). To our knowledge, the rate of ADP- ribosylation in aging brain has not yet been reported. Since different types of

the cell populations in the brain, such as neurons and astroglias, are known to have varied functional[22] and metabolic activities,[25] and because DNA is directly involved in ADP- ribosylation, it would be of interest to investigate the alteration of ADP- ribosylation and the rate of DNA breaks in the neuronal and astroglial cells during brain aging. The results presented in this report reveal marked differences between ADP- ribosylation and the rate of DNA breaks of the neurons and astroglials in the adult and aged rat brain.

MATERIAL AND METHODS

Animals

Male albino rats of the Wistar strain were used and fed *ad libitum* and drinking water was always available. The ages of the animals were carefully noted from birth. Adult rats were three months old and aged rats were 30 months old.

Cell isolation

Cells were isolated from rat brain according to the method of Johnson and Sellinger.[20] In each experiment, three rats were killed by decapitation and brains were removed onto an icecooled glass plate over crushed ice and chopped into the consistency of a mince, which was rapidly transferred into 30-35 ml of an ice- cold solution of 7.5% (w/v) polyvinylpyrrolidone (PVP, mol. wt. 40,000, 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 110μm pore size nylon mesh. The cell suspension was then passed three times through 80μm 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 41, 000 g for 30 min. Purified neurons were obtained as a pellet in 1.75 sucrose, and the glial cells 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. Each cell type retained its characteristic appearance and shape and was easily recognised.[10,20] Some broken cells were seen in the astroglial fractions.

Enzyme- linked immunosorbent assay

In order to determine the relative purity of the cell fractions, enzyme- linked immunosorbent assays (ELISA) were performed to assess the nurofilament and glutaminesynthetase contents of the cell fractions. The ELISA was performed on the frozen- thawed cellular fractions diluted to 25 μg/ml in 50 mM bicarbonate buffer pH 8.5. In each immunoplate (NUNC) well, 200 μl of the homogenate 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. Primary antibody (anti- neurofilament, Pierce, or anti- glutamine synthetase produced in Centre de Neurochimie) 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 and then incubated with a 1:500 dilution of rabbit anti- mouse IgG peroxidase conjugated (Pierce) for 2h at room temperature. After three final wash steps, the wells were incubated with 50 mM 5- amino- salicylsaure (OSI Distributeur) and 5 mM H_2O_2 in a 0.1 M sodium acetate solution pH 5.5. The reaction was stopped with 100μl sodium hydroxide and the absorbance of each well was determined 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 100μg/ml protein. Duplicate wells were prepared for each sample, and the mean absorbance was used in subsequent calculations. On each plate, standards of neurofilaments (low molecular weight, BM68) or glutamine synthetase (sheep brain, Sigma) in a range concentration of 50 to 1000 ng/ml were used.

Assay of ADP-ribosyl transferase

ADP- ribosyl transferase activity was assayed in an incubation mixture of 10 mM Tris/HCl pH 8.4 mM $MgCl_2$, 0.4 mM dithiothreitol and 0.1 mM [^{3}H]NAD (10,000CPM/nmol). The reaction was started by addition of 40 μl of (200 μg protein) cellular fraction and carried out for 10 min at 37°C in a total volume of 125 μl. 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 4°C, the precipitate was collected on a Whatman GF/B glass fiber paper and 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 enzyme which catalyzes the incorporation of 1 pmol of ADP- ribose into the acid- insoluble material per 10 min at 37°C.

Determination of DNA strand breaks

DNA strand breaks were measured fluorimetrically as described by Birnbim and Jevcak.[4] The cell lysates

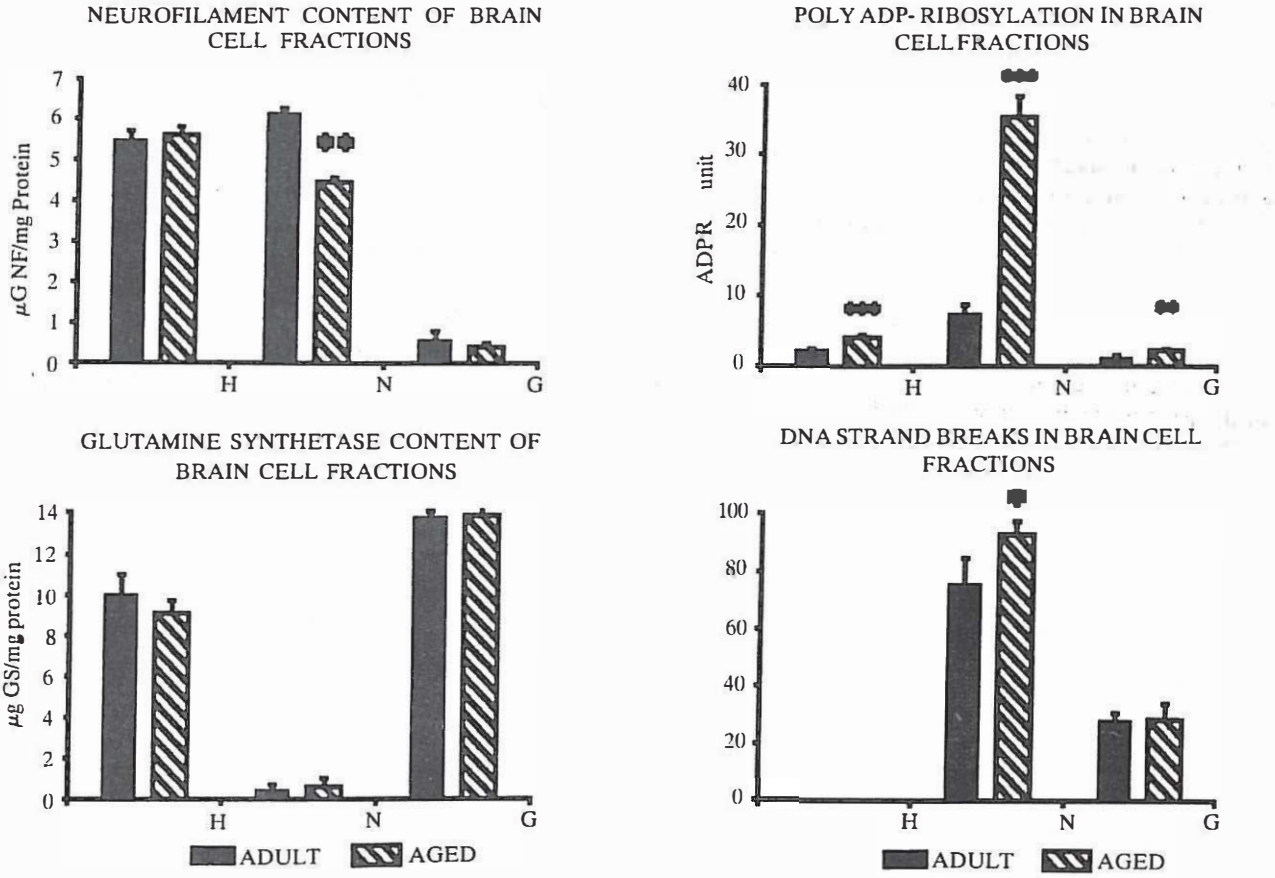

Fig. 1. Effects of age on the neurofilament, glutamine synthetase, poly ADP- ribosylation and DNA breaks in brain homogenate (H), neuronal (N) and astroglial (G) cell fractions. ELISA was performed for the determination of the neurofilament and glutamine synthetase using specific antibodies and IgG peroxidase conjugated. ADP-ribosylation was assayed by the incorporation of [3H] DNA into proteins, followed by scintillation counting (see Materials and Methods). The amount of DNA breaks was estimated fluorometricaly as described by Birnboim and Jevcak (1981).

were exposed to alkaline solution and the rate of strand unwinding was determined directly using ethidium bromide (6.7 μg/ml 13.3 mM NaOH) solution as a fluorescent dye. The fluorescence was monitored at excitation of 520 nm and emission of 590 nm.

Protein and DNA measurement

Protein was assayed by the method of Lowry, et al,[29] with bovine albumin as the standard. DNA was measured by the fluorometric assay of Labarca and Paigen.[24]

RESULTS

The data depicted in Fig.1 can be analysed from four different perspectives: (a) the relative purities of the cellular preparations; (b) the relative activity of poly ADP- ribosyl polymerase in the neuronal and astroglial cells; (c) the relative rate of DNA breaks in the cellular fractions; and (d) any differences that may be evident that are related to the age of the animals under study. In agreement with a previous report by Langley, et al,[26] the neurofilament polypeptide is exclusively concentrated in the neuronal fraction (Fig.1). Although the peptide measured in the brain homogenates do not seem to alter to any extent during aging, however a significant decrease of the peptide was recorded in neuronal fraction of the aged rat brain. The neurofilament content of the astroglial cells do not show a significant change in the aged rat brain though there are indications of lower values compared with the adult rat brain astroglial fraction. In contrast, glutamine synthetase is exclusively concentrated in the astroglial fractions with no alteration during aging, although slight decrease of the enzyme was noted for the brain homogenate, as compared to that of adults.

These cellular preparations were used to investigate poly ADP- ribosylation in the aging brain. The results are also demonstrated in Fig.1. When poly ADP-

ribosyl polymerase activity was measured in rat brain cell suspensions (before sucrose gradient cell separation) an approximately two- fold increase of the enzyme activity was observed in the aged rat brain. There is however a significant increase of about five- fold in poly ADP- ribosylation of the neuronal fractions in aging brains, when compared with the adult brains. The enzyme activity of the astroglial fractions prepared from adult rat brain was much lower than that of the neuronal fractions. There is however an increase of about 67% in the poly ADP- ribosylation of the aging brain astroglias when compared with the adult cells.

The amount of DNA breaks was also estimated in the neuronal and astroglial fractions with respect to age. As can be seen in Fig.1, the neuronal fraction contained a relatively high amount of single- stranded DNA (170%) as compared with the astroglial cells. Aging resulted in an increase of about 12% in the amount of breaks in the neurons, whereas no change was seen in the amount of DNA breaks in the aged astroglial cells.

DISCUSSION

This paper reports that poly ADP- ribosyl polymerase- specific activity of the aged rat neuronal cells is some five- fold higher than that of the adult rat neuronal fractions. It is rather intriguing since most studies seem to agree that the activity of many enzymes decrease in the aging brain. The difference in the values of ADP- ribosylation in the adult and aged neurons could probably be explained by higher levels of DNA breaks in the neuronal cells. Although there may be some kind of artifact cell damage during cell separations, the relative purity of the cell fractions as judged by high neurofilament content of the neurons and high glutamine synthetase content of the astroglial cells was adequate to investigate the effect of aging on neuronal and non- neuronal cells. The results indicated that in parallel to increasing the rate of ADP- ribosylation, aging increased the rate of DNA breaks in the neurons but not in the astroglial preparations (see Fig.1). Because the nuclear DNA and ADP- ribosylation of nuclear proteins are obviously implicated in the same nuclear processes,[30] 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 the cells are tightly associated with the stuctural integrity of nuclear DNA.[19,37] It is becoming increasingly clear now that DNA in aging brain cells may accumulate considerable damage. Previous studies have shown the accumulation of an increasing amount of single- stranded regions in the DNA with age.[8] However, higher levels of DNA breaks in the neurons as compared to that in the astroglial cell fractions may be due, at least in part, to different metabolic activities of the brain cell populations. In the neurons, oxygen consumption appears to be higher than that of other cell types.[25] Oxygen metabolism is known to generate oxygen- derived free radicals, which if accumulated can induce tissue damage.[15,23,35] It has been reported that agents which provoke DNA damage or strand breaks produced an activation of the ADP- ribosyl polymerase activity.[31] Furthermore it has been postulated that aging may be due to free radical reaction damage.[16] Thus, unexpected high levels of ADP- ribosylation in the neurons and much lower values in the astroglials, having a close relationship with the rates of DNA breaks of the cellular fractions, suggest that the neurons are more susceptible to the metabolic alterations of the aging process.

In conclusion, the data reported here lend credence to the possibility that different cell types of the brain may be affected differentially by aging.

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