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ORIGINAL PAPER

Oxidative Damage in Rat Brain During Aging: Interplay Between Energy and Metabolic Key Target Proteins

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Abstract Aging is characterized by a gradual and continuous loss of physiological functions and responses particularly marked in the central nervous system. Reactive oxygen species (ROS) can react with all major biological macromolecules such as carbohydrates, nucleic acids, lipids, and proteins. Since proteins are the major components of biological systems and regulate multiple cellular pathways, oxidative damage of key proteins are considered to be the principal molecular mechanisms leading to age-related deficits. Recent evidences support the notion that a decrease of energy metabolism in the brain contribute to neuronal loss and cognitive decline associated with aging. In the present study we identified selective protein targets which are oxidized in aged rats compared with adult rats. Most of the oxidatively modified proteins we found in the present study are key proteins involved in energy metabolism and ATP production. Oxidative modification of these proteins was associated with decreased enzyme activities. In addition, we also found decreased levels of thiol reducing system. Our study demonstrated that oxidative damage to specific proteins impairs energy metabolism and ATP production thus contributing to shift neuronal cells towards a more oxidized environment which ultimately might compromise multiple neuronal functions. These results further confirm that increased protein oxidation coupled with decreased reducing systems are characteristic hallmarks of aging and aging-related degenerative processes.

Keywords Protein oxidation · Aging · ATP synthesis · Carbonylation

Introduction

Aging is a complex process involving alterations at genetic, molecular and cellular levels [1]. Growing body of evidence indicates that reactive oxygen species (ROS) are one of the primary determinants of aging. The "oxidative stress theory" of aging implicates that a progressive and irreversible accumulation of cellular and tissue oxidative damage caused by ROS impacts on critical aspects of the aging process and contributes to impaired physiological function, increased incidence of disease and a reduction in life span [2, 3]. ROS are highly reactive molecules towards all cellular macromolecules such as DNA, lipids and proteins and the accumulation of oxidative damage over the lifetime is a characteristic hallmark of the aging process [4–6].

Oxidative stress alters the cell redox status thus leading to the chemical modifications of target proteins that may be associated with loss of protein function, caused either by reversible or irreversibile reactions [7]. Major molecular mechanisms leading to structural changes in proteins are metal-catalyzed protein oxidation characterized by protein carbonyl formation [8, 9], loss of protein thiol groups,

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presence of nitrotyrosine [9, 10], HNE modifications and advanced oxidation protein products formation [11].

Attack by ROS upon proteins can damage several amino acid residues, including histidine, proline, arginine and lysine. Oxidative damage to these amino acid residues and/or to the peptide backbone of proteins can generate protein carbonyl products. Compared to other different types of post-translational modifications, carbonylation is an irreversible oxidative process [12] that requires the proteasome system to be removed [13]. Different sensitive methods have been developed for the detection and quantification of protein carbonyl groups and most of these involve derivatization of the carbonyl group with 2, 4-dinitrophenol hydrazine and subsequent immunodetection of the resulting hydrazone using monoclonal or polyclonal antibodies [14]. Redox proteomics approach has been developed [15] to identify specific proteins which show increased carbonylation. By following this approach, several proteins have been identified to be oxidized in the brain from mild cognitive impairment and Alzheimer's disease subjects compared with controls [16]. It appears that such modifications target very specific proteins and can affect the integrity and functioning of the proteome with relevant implications in the pathogenesis and progression of Alzheimer's disease and other neurodegenerative disorders [15, 17].

Previous studies have demonstrated that oxidative processes can modulate biochemical characteristics of protein such as enzymatic activity often resulting in decreased activity of key enzymes including glutamine synthetase (GS), creatine kinase (CK) and tyrosine hydroxylase [18–20].

Recently, we have identified by redox proteomics approach several proteins in different brain region of senescent compared with aged rats which are involved in ATP production, cell structure, antioxidant response and cell signalling [21]. In the present study we provide further evidence on the effects of oxidative damage on protein function. As an effect of oxidative modifications, loss of activity of our identified proteins directly, or indirectly, involved in energy metabolism and ATP production, may contribute to abnormal energy production and, as such, be relevant to pathogenesis of brain aging process.

Experimental Procedure

Animals and Samples Preparation

All animal protocols were approved by the University of Catania Laboratory Animal Care Advisory Committee. Male Wistar rats purchased from Harlan (Udine, Italy) were maintained in a temperature and humidity-controlled room with a 12 h light: dark cycle. Rats ($n = 8$, per group) of 12 (aged) and 28 (senescent) months, were fed ad libitum. After sacrifice, brains were quickly removed and dissected into the cerebral hippocampus, cortex, cerebellum, and striatum according to a standardized procedure, in a cold anatomical chamber and following a protocol that allows a maximum of 50 s time-variability for each sample across animals. Brain samples from hippocampus, cerebellum, striatum and cortex were minced and suspended in 10 mM HEPES buffer (pH 7.4) containing 137 mM NaCl, 4.6 mM KCl, 1.1 mM KH_2PO_4, 0.1 mM EDTA, and 0.6 mM $MgSO_4$ as well as proteinase inhibitors: leupeptin (0.5 mg/ml), pepstatin (0.7 μg/ml), type II S soybean trypsin inhibitor (0.5 μg/ml), and PMSF (40 μg/ml). Homogenates were centrifuged at 14,000 × g for 10 min to remove debris. Protein concentration in the supernatant was determined by the "Coomassie Plus Protein Assay" (Pierce, Rockford, IL, USA).

Enzyme Assays

ATP Synthase

Mitochondrial ATP synthase activity was measured spectrophotometrically at 340 nm by coupling the production of ADP to the oxidation of NADPH via the pyruvate kinase and lactate dehydrogenase reaction (coupled assay) as previously described [22]. The reaction mixture (0.2 mL final volume) contained: 100 mM Tris (pH 8.0), 4 mM Mg-ATP, 2 mM $MgCl_2$, 50 mM KCl, 0.2 mM EDTA, 0.23 mM NADH, 1 mM phosphoenolpyruvate, 1.4 unit of pyruvate kinase, 1.4 unit of lactate dehydrogenase, and about 25–50 μg of proteins (brain homogenates), and was assayed at 30°C. The assay was carried out in a microtiter plate reader (Labsystem Multiscan MS).

Adenylate Kinase

Adenylate kinase (AK) activity was measured with a coupled enzyme assay with hexokinase (HK) and glucose 6-phosphate dehydrogenase (G6PD), according to Oliver [23] with the modifications introduced by Dezja et al. [24]. The reaction mixture contained 100 mM KCl, 20 mM HEPES, 20 mM glucose, 4 mM $MgCl_2$, 2 mM $NADP^+$, 1 mM EDTA, 4.5 U/ml of HK, 2 U/ml of G6PD and 1 μg of protein homogenate. The reaction was initiated by the addition of 2 mM ADP and the reduction of $NADP^+$ was followed at 340 nm for 3 min in a spectrophotometer. ADP, $NADP^+$, cystine, G6PD and HK were dissolved in water. Reagents concentration and assay time (3 min) were chosen to assure the linearity of the reaction. The results were expressed in μmol of ATP formed per min per mg of protein.

Creatine Kinase

Creatine kinase (CK) enzyme activity was assayed according to [25]. The reaction mixture contained the following final concentrations: 60 mM Tris-HCl buffer (pH 7.5), 7 mM phosphocreatine, 9 mM MgSO4, 0.02% triton X-100, and approximately 1 μg protein in a final volume of 0.1 mL. After 15 min of pre-incubation at 37°C, the reaction was started by the addition of 0.3 μmol ADP plus 0.08 μmol reduced glutathione. The reaction was stopped after 10 min by the addition of 1 μmol p-hydroxymercuribenzoic acid. The reagent concentrations and the incubation time were chosen to assure linearity of the enzymatic reaction. The creatine formed was estimated according to the colorimetric method of Hughes (1962). The color was developed by the addition of 0.1 mL 2% naphtol and 0.1 mL 0.05% diacetyl in a final volume of 1 mL and read after 20 min at 540 nm. Results were expressed as micromol of creatine formed per min per mg protein.

Triosephosphate Isomerase

Triosephosphate isomerase (TPI) activity was assayed as described previously [26]. Brain homogenate (10 μl, 0.1 μg/μl) was mixed with 190 μl of assay solution (20 mM triethanolamine-HCl buffer, pH 7.9, 300 μM NADH, 0.4 mM d-glyceraldehyde 3-phosphate, 40 μg/ml glycerophosphate dehydrogenase) in a UV-transparent microtiter plate. TPI activity was assessed by the decrease in A_{340} at 25°C for 3 min. Three measurements were taken for each sample.

Malate Dehydrogenase

Malate dehydrogenase (MDH) activity was measured spectrophotometrically at 340 nm by following the rate of the oxidation of NADH in the presence of oxaloacetate. The reaction mixture (0.2 ml final volume) contained: 2 mM oxaloacetic acid in 100 mM potassium phosphate buffer, pH 7.5, 0.14 mM NADH and 5 μl of protein sample. The assay was carried out at 25°C in a microtiter plate reader (Labsystem Multiscan MS).

Total Sulfhydryl (SH) Groups Assay

Protein and non protein sulfhydryl compounds in different brain regions were estimated by the DTNB-based method of Sedlak and Lindsey [27]. The content of SH groups was expressed in nmol per mg of protein.

Statistical Analysis

Results were expressed as means ± SEM of at least eight separate experiments. Statistical analyses were performed using the software package SYSTAT (Systat Inc., Evanston IL, USA). The significance of the differences, evaluated by two-way ANOVA, followed by Duncan's new multiple-range test, was considered significant at $P < 0.05$. The significance of the change in carbonylation of specific proteins in the proteomics study was evaluated via non-parametric Mann-Whitney-Wilcoxon test. $P < 0.05$ was considered statistically significant.

Results

Protein Oxidation in Senescent vs Aged Rats

Extending our recent study on protein carbonylation in the brain of senescent rats [21], we have used a parallel approach to quantify the protein levels by Coomassie staining and the carbonyl levels by immunohistochemistry. The specific carbonyl levels were obtained by dividing the carbonyl level of a protein spot on the nitrocellulose membrane by the protein level of its corresponding protein spot on the gel. Comparative Image analysis by PdQuest software (Bio-Rad) allowed the identification of specific spots that showed increased carbonyl levels in senescent vs aged rat. These spots were manually excised from the gels and identified by MALDI-ToF mass spectrometry [21].

In the present study we focused on specific proteins, mostly involved in energy metabolism and ATP production. Figure 1 (2D gel at the top, 2D blot at the bottom) shows the proteins, involved in energy metabolism, which resulted significantly more oxidized in the cortex from senescent compared with aged rats: Creatine kinase B-type, Fructose-bisphosphate aldolase A, Vacuolar ATP synthase subunit B, Pyruvate kinase isozymes M1/M2, ATP synthase subunit alpha and Triosephosphate isomerase.

In senescent rat striatum we identified Adenylate kinase, Pyruvate kinase isozymes M1/M2, Aconitate hydratase, Triosephosphate isomerase and Glyceraldehyde-3-phosphate dehydrogenase 5 proteins to be significantly more oxidized than in aged rats.

In senescent rat hippocampus these oxidatively modified proteins are: Creatine kinase, malate dehydrogenase, α-enolase, pyruvate kinase, fructose-bishosphate aldolase C and ATP synthase subnit alpha.

In senescent rats cerebellum Malate dehydrogenase, Isocitrate dehydrogenase, Pyruvate kinase isozymes M1/M2, Phosphoglycerate kinase 1 and Adenylate kinase isoenzyme 4 showed increased carbonyl levels compared with aged rats.

All the oxidatively modified proteins are listed in Table 1 with their location and function.

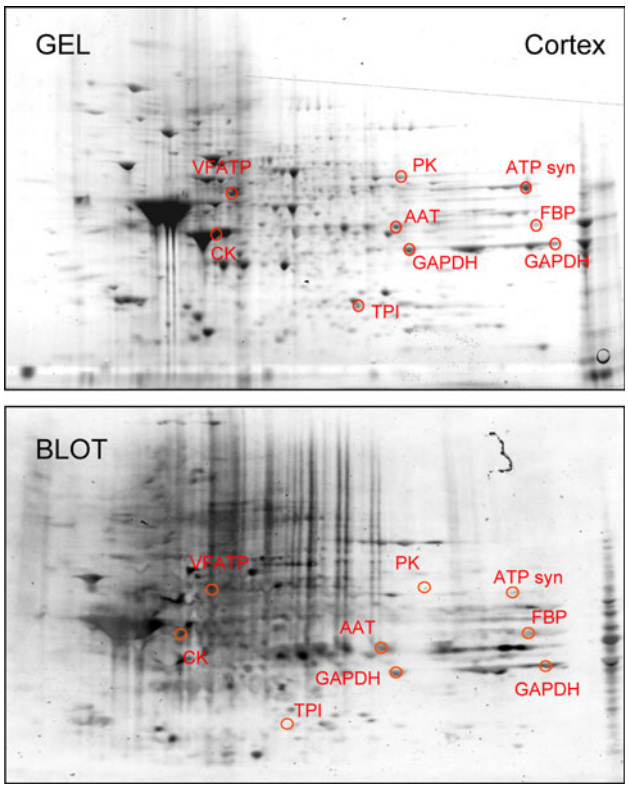

Fig. 1 Representative 2-D gels of aged rat cortex. Proteins (200 μg) were separated on immobilized pH 3–10 IPG strips followed by separation on 8–16% gradient SDS-PAGE gels and stained with Biosafe Coomassie (*top*). Rapresentative 2-D oxyblot from rat cortex. Relative change in carbonyl immunoreactivity, after normalization of the immunostaining intensities to the protein content, was significant for six proteins (*bottom*). See text

Enzyme Activity

Several studies from our laboratory and others demonstrated that protein oxidation likely lead to protein dysfunction [17, 28, 29]. Most of the oxidative post-translational modifications are irreversible and they are responsibile of the impairment of multiple cellular functions. Therefore, we measure the enzyme activty of some of the enzymes we found oxidized with the redox proteomics approach. Specifically, ATP synthase and CK were found to be decreased in hipocampus and cortex, AK in cerebellum and striatum of senescent vs aged rats (Fig. 2). In addition, the enzyme activity of TPI in cortex and striatum was found to be significantly decreased in senescent rats compared with aged rats (Fig. 3). Conversely, MDH activity was increased in senescent vs aged rat hippocampus while no significant differences were observed in cerebellum (Fig. 4). This result is in agreement with previous findings showing an increase of MDH activity with aging [30].

Total Sulfhydryl Groups Analysis

Intracellular thiols were investigated in different brain regions as a function of age. As shown in Fig. 5 total sulfhydryl groups decreased significantly in all cerebral areas examined except cerebellum. Approximately a 30% decrease was found in hippocampus, striatum and cortex of 28 months compared to adult 12 months old animals.

Discussion

Aging is characterized by a general decline in physiological functions that affects expecially the brain which is particularly susceptible to the effects of oxidant injury. Thus, impaired function of the central nervous system in aged animals is associated with increased susceptibility to the development of many neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD),

Table 1 List of all the proteins identified to be oxidatively modified in the four brain regions with their location and function

Proteins	Brain region	Location	Function
GAPDH	Striatum, Cortex	Cytoplasm	Glycolysis
PGK	Cerebellum	Cytoplasm	Glycolysis
PK	Cerebellum, Striatum, Cortex, Hippocampus	Mitochondria, Cytoplasm	Glycolysis
TPI	Cortex, Striatum	Cytoplasm	Glycolysis
α-enolase	Hippocampus	Cytoplasm	Glycolysis
FBP	Cortex, Hippocampus	Cytoplasm	Glycolysis
VFATP	Cerebellum	Cytoplasm	Energy metabolism
CK	Cortex, Hippocampus	Cytoplasm	Energy metabolism
AK	Cerebellum, Striatum	Mitochondria, Cytoplasm	Energy metabolism
ATP syntase	Cortex, Hippocampus	Mitochondria	Energy metabolism
ISDH	Cerebellum	Mitochondria	Krebs cycle
MDH	Cerebellum, Hippocampus	Mitochondria	Krebs cycle
ACO	Striatum	Mitochondria	Krebs cycle

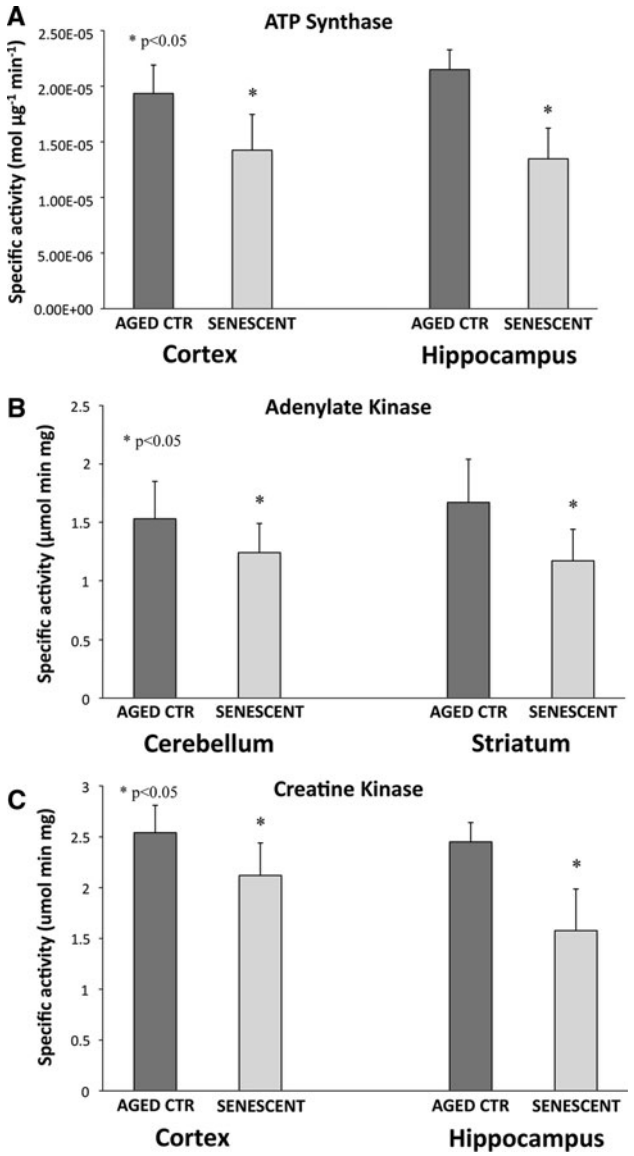

Fig. 2 Enzyme activity of ATP synthase **a**, AK **b** and CK **c** in all the brain regions of senescent rats compared with aged controls. Enzyme activity of ATP synthase, CK and AK are significantly reduced in senescent vs aged rats ($P < 0.05$). *Bars* represent mean ± S.E.M; $n = 8$ for each group

and amyotrophic lateral sclerosis (ALS) [31, 32]. The levels of oxidatively damaged proteins increase with age [17, 33, 34], with a parallel loss of protein sulfhydryl groups and reduced activity of important metabolic enzymes.

Recently, we identified the proteins that showed increased carbonyl levels in four different brain regions of 28 months old rats compared with 12 months old rats [21]. Our results showed the oxidation of a large number of proteins involved in energy metabolism, including ATP production, glycolysis and Krebs cycle. Oxidation of these proteins was accompained by reduced enzyme activity.

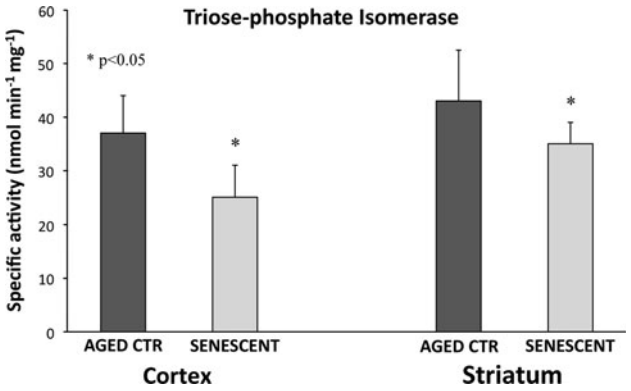

Fig. 3 Activity of TPI in cortex and striatum of senescent rats compared with aged control animals. TPS enzyme activity is reduced in senescent vs aged rats ($P < 0.05$). *Bars* represent mean ± S.E.M; $n = 8$ for each group

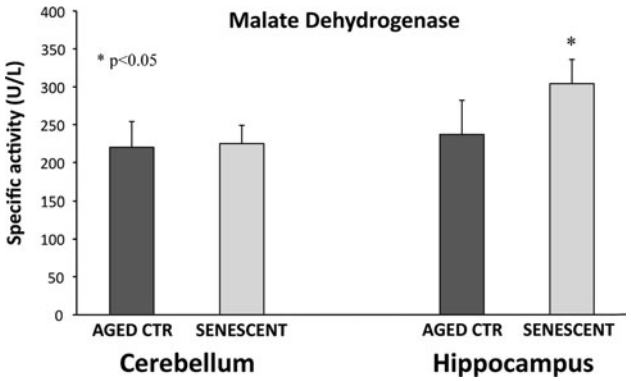

Fig. 4 Activity of MDH in hippocampus and cerebellum of senescent rats compared with aged control animals. An increase of MDH enzyme activity was measured in senescent vs aged rats in hippocampus while no differences were observed in cerebellum ($P < 0.05$). *Bars* represent mean ± S.E.M; $n = 8$ for each group

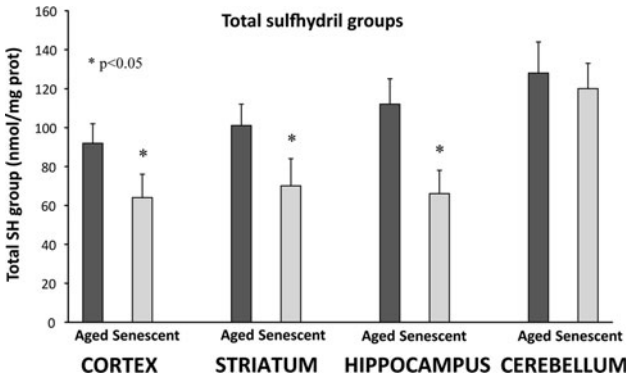

Fig. 5 Total sulfhydryl groups decreased significantly in hippocampus, striatum and cortex of senescent vs aged rats (30%). Whereas in cerebellum no statistically significant differences were found between

These results further support the notion that oxidative damage of specific cellular targets impair cell functionality thus contributing to increased neuronal loss. Accumulation

of oxidative damage together with decreased levels of thiol reducing system may contribute to the development of age-related degenerative phenomena. Reduction of sulfhydryls groups was also associated with a reduction of both cytosolic and mitochondrial GSH and a parallel increase of GSSG in different brain regions of senescent vs aged rats [21]. Oxidative stress conditions are responsible of reduced sulfhydryls leading to decreased reducing environment of the cell. This redox shift may increase cellular susceptibility to accumulate oxidative damage. Indeed, oxidative stress results from the imblance between increased production of ROS and decreased antioxidant capcacity of the cell, i.e. reducing systems.

Here we describe the specific function of carbonylated proteins, which showed impaired enzyme activity, with their implication in energy metabolism and ATP production.

Energy Metabolism Proteins

We found a significant increase in the oxidation of several *Gyicolytic* enzymes, these are: Fructose-bisphosphate aldolase (FBP), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Phosphoglycerate kinase 1 (PGK1) and triosephosphate isomerase (TPI).

GAPDH undergoes significant nitration, another form of oxidative modification, in the hippocampus of AD patients and also in rats after intracerebral injection with Amyloid-β (1–42) [35, 36]. Recent studies have demonstrated that oxidative inactivation of GAPDH may be involved in neurotoxicity [36]. We showed decreased enzyme activity of GAPDH in cortex and striatum of senescent vs aged rats [21]. Indeed, studies from other groups showed that oxidative modification of this enzyme is associated with a decrease of its activity during aging [37, 38].

TPI catalyzes the reversible interconversion of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. Previous studies on SAMP8 mice showed an increase of oxidation of TPI but no change in enzyme activity was observed [38]. Here we found that increased carbonylation of TPI was also associated with decreased enzyme activity. The inhibition of TPI contribute to decreased neuronal ATP production followed by progressive neuronal death [39].

PK catalyzes the final step in glycolysis, with the concomitant transfer of the high-energy phosphate group from phosphoenolpyruvate to ADP, thereby generating ATP. This enzyme controls the flow of glucose into synthetic pathways or into metabolism, so it is crucial to the energy regulation of the cell [37]. Other studies report an impairment of PK, caused by oxidative damage, in mild cognitive impairment [40] or Down syndrome [41].

Taken toghether, these results support the idea of the involvement of glycolysis failure as one of the first event associated with aging progression. In fact impairment of glycolysis, as indicated by the reduced activity of the enzymes listed above, could lead to decreased ATP production observed in aging brain. A decrease in ATP production would accordingly lead to dysfunction in electrochemical gradients, ion pumps, and voltage-gated ion channels, altering membrane cell potential [36].

In our model of aging also *the Krebs cycle* turned out as a target of oxidation. Among the identified enzymes those which showed increased carbonyl levels were isocitrate dehydrogenase (ISDH), malate dehydrogenase (MDH) and aconitase.

ISDH catalyzes the first oxidative conversion in the TCA cycle: isocitrate and NAD^+ are converted to α-ketoglutarate, NADH and CO_2. Isocitrate dehydrogenase is a highly regulated enzyme. The enzyme is stimulated by NAD^+ and ADP and inhibited by NADH, making it very sensitive to the NADH-to- NAD^+ ratio [42].

MDH is also essential to energy production and regulation. This enzyme is involved in the malate–aspartate shuttle catalyzing the conversion from malate to oxaloacetate in glicolysis, also producing ATP from ADP. MDH is located within the mitochondrial matrix in order to connect glycolysis to mitochondrial respiration. This protein transfers NADH across the mitochondrial membrane to respiratory complex I [43]. Here we showed that enzyme activity of MDH is increased in senescent versus adult rat hippocampus. These data confirm previous studies showing an increase of MDH during aging [30].

Aconitase an iron-sulfur protein of the mitochondrial matrix catalyzes the stereospecific isomerization of citrate to isocitrate via *cis*-aconitate. The particular susceptibility of mitochondrial aconitase to oxidative damage may be related to the iron-sulfur cluster [4Fe-4S] in its active site [44]. Age-related oxidative inhibition of this enzyme has already been observed and may block normal electron flow to oxygen, leading to an accumulation of reduced metabolites such as NADH [44, 45]. Collectively, the oxidation of TCA enzymes and the consequent impairment of their activity may affect ATP levels.

We also found the oxidation of others proteins directly involved in ATP production such as ATP synthase, Adenilate kinase (AK) and creatine kinase (CK).

ATP synthase is localized in the inner membrane of mitochondria and is a part of the complex V that plays a key role in energy production. We found increased carbonyl levels and reduced enzyme activity of ATP synthase in the cortex and hippocampus of senescent rats. Dysfunction of ATP synthase could contribute to a decrease in the activity of the entire electron transport chain and impaired ATP production [46, 47]. The proteomic identification of ATP synthase impairment in senescent rats brain provides further evidence of the role of mitochondria in the aging process.

AKs are ubiquitous enzymes which are involved in maintaining the homeostasis of adenine and guanine nucleotide composition in various organisms. In the central nervous system, ATP and GTP are thought to be not only the energy source but also the principal neurotransmitter or neuromodulator at purinergic synapses. Furthermore, it has been found that there is substantial AK activity in developing mouse brain during the period of rapid neural growth and it has been concluded that AK is involved in neural functioning. We found an increased oxidation of AK in cerebellum and striatum of senescent rats compared to aged. This increased oxidation correlate with decreased enzyme activity and subsequently to the loss of adenine and guanine nucleotide homeostasis in the cell.

CK system is the most important immediate energy buffering and transport system especially in muscle and neuronal tissue [48]. Creatine is phosphorylated to phosphocreatine in the intermembrane space of mitochondria where mitochondrial CK is located and is then transported into the cytosol. There the energy pool can be regenerated by transphosphorylation of phosphocreatine to ATP, which is catalyzed by cytosolic CK located in close vicinity of cellular ATPases [48, 49]. CKs are prime targets of oxidative damage, and is also well established that oxidative modification of CK-BB decreases its activity in aging, such as in the brain of old brown Norway rats, in AD and other neurodegenerative diseases [44, 50, 51]. Consistent with these studies we show increased oxidative modifications of CK-BB monomer and decreased enzyme activity in the cortex of senescent rats. In aging rats and humans there is a decrease in the levels of cardiac CK activity [52]. Age-related declines in GS and CK activities have been observed in gerbil and human brain tissues [28, 53].

Considering that CK and AK are intimately associated in such a way that when one enzyme activity is reduced, the activity of the other enzyme is enhanced [54], the simultaneous inhibition of the two enzymes could disrupt energy homeostasis with severe consequences for cell function and survival. Collectively, diminished activity of PK, CK and AK leads to a severe reduction of ATP content with severe implication of neuronal functionality.

Neurons require high levels of ATP in order to sustain various neuronal processes such as firing of action potentials, neurotransmission and ion homeostasis [55]. As such, age-related decline in metabolism may contribute to the cognitive declines associated with aging [2] and which may be further exacerbated in selective regions of the brain in AD and PD subjects [56, 57].

In conclusion, the present study show that a number of proteins involved in energy metabolism pathways are significantly more oxidized in the brain of senescent versus aged rats. The loss of the activities of these proteins by oxidative modification may contribute to abnormal energy production that has been shown as one of the primary event involved in aging and aging-related neurodegenerative disorders. Our data further confirm that the failure of energy metabolism is a feature of aging and also contributes to the pathophisiology of many neurodegenerative diseases.

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