Vol. 199, No. 2, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 15, 1994 Pages 726-733

INHERITED ENHANCEMENT OF HYDROXYL RADICAL GENERATION AND LIPID PEROXIDATION IN THE S STRAIN RATS RESULTS IN DNA REARRANGEMENTS, DEGENERATIVE DISEASES, AND PREMATURE AGING

R.I.Salganik, N.A.Solovyova, S.I.Dikalov, O.N.Grishaeva, L.A.Semenova, and A.V.Popovsky*

Institute of Cytology & Genetics and * Institute of Kinetics & Combustion, Siberian Branch of the Russian Academy of Sciences, 630090 Novosibirsk, Russia

Received January 25, 1994

Summary: Previously by selection and inbreeding of Wistar rats susceptible or resistant to the cataractogenic effect of galactose the S and R rat strains differing in the intensity of hexose transport into the animal cells were developed. High level of OH-radical generation and enhanced lipid peroxidation are revealed in the liver and myocardium of the S rats in contrast to the R rats. Data are obtained supporting the view that enhanced generation of OH-radicals within the S rat tissues is due to oxidation and autooxidation of the abundant amounts of monosaccharides intensely accumulating in the rat cells. In spite of continuous inbreeding for more than 40 generations and a high rate of homozygosity, numerous DNA rearrangements are revealed in the S rat genomes. Fragility of the S rat cell membranes is detected. Cataracts and other lens lesions, emphysema, tumors, cardiomyopathy-like changes in the myocardium, scoliosis, brain disfunctions are characteristic of the S rats, as well as low fertility and short life-span indicative of premature aging.

Many lines of evidence support the view that oxidative damage of DNA, proteins, lipids of biomembranes and lipoproteins is involved in a number of disease states including cardiovascular diseases, arthritis, cataract, emphysema, brain disfunction, very likely, cancer and results in degenerative processes leading to aging [1-4]. Endogenous oxidants arising as by-products of normal metabolism include superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\cdot}OH$). A rise in the generation of endogenous oxidants due to the changes in metabolic processes may lead to enhancement of their damaging effects increasing thereby manifestations of pathological conditions.

Availability of an animal strain with continuously enhanced generation of endogenous oxidants would help to reveal their role in pathology, to study the ef-

0006-291X/94 $5.00

ficiency of antioxidants and other means of prevention and treatment of the oxidant-caused diseases.

Previously by selection and inbreeding of Wistar rats highly susceptible or resistant to the cataractogenic effect of galactose-rich diet a sensitive (S) and a resistant (R) rat strains were developed [5]. It was found that a high level of hexose transport into the animal cells due to the enhanced specific activity of glucose transporter is characteristic of the S rats [6]. In the present paper we show that an enhanced hydroxyl radical generation and lipid peroxidation in the S rat tissues occur as stably expressed inherited features. Additional administration of hexose to animals results in increase in $^{\cdot}OH$ generation and lipid peroxidation. These data support the view that hyperproduction of free radicals and enhanced lipid peroxidation are the results of increased oxidation and autooxidation of the abundant monosaccharides [7-9]. In spite of strict inbreeding for more than 40 generations and high rate of homozygosity there are frequent rearrangements of the DNA found in the S rat genomes. Lesions of cellular membranes in S rats obviously due to the enhanced lipid peroxidation are demonstrated by the fragility of lysosomes resulting in the outflow of the lysosomal hydrolases. Premature aging and a number of morbid conditions are observed in S rats: cataracts, emphysema, scolyosis, tumors, alterations of myocardium resembling human cardiomyopathy and long-term memory impairment. Premature aging and short life-span are characteristic of the S rats. In control rats of the R strain no increase in free radical generation, lipid peroxidation, DNA rearrangements and manifestations of the morbid conditions were found.

MATERIALS AND METHODS

Adult male rats of the S and R strains (Breeding Laboratory of the Institute of Cytology and Genetics, Novosibirsk, Russia) at the age of 2 to 12 months were used throughout. The S rats were selected for their high sensitivity to the cataractogenic effect of galactose-rich diet. They have been previously accepted as models of galactosemia and were designated as W/SSM rat strain [5]. The R rats were selected for their resistance to the cataractogenic effect of high doses of galactose. The S and R rat strains were maintained by strict inbreeding for 42 generations.

The rats were killed by decapitation. Liver and heart homogenates and mitochondria were prepared as described [10]. Generation of $^{\cdot}OH$-radicals was studied by electron spin resonance spectroscopy (ESR) using the spin trap 2,2-dimethylpyrroline-N-oxide (DMPO, Sigma), forming with $^{\cdot}OH$ a spin adduct, DMPO-OH. ESR spectra were measured by ER-200D SRC spectrometer (Bruker) in a 200 ml flat sealed quartz cell under the following conditions: the center of field 3474 G, micro-wave power 20 mW, modulation amplitude 1G, gain $5x10^5$. The DMPO was purified according to [11]. In OH-radical measurements the samples contained 50μm 2-dimethylamino-3-chlor-1,4-naphtoquinone, 0.1 M DMPO, 1 mg protein per ml of rat liver or heart homogenate or mito-

chondria, 1 mM NaN_3, 0.1 mM H_2O_2in buffer containing 50 mM Tris-HCl, 0.1 M KCl (pH 7.4).

Lipid peroxidation was measured with the thiobarbituric acid (TBA) assay as described [12] and expressed as the absorbance at 535 nm vs 600 nm ($\Delta A_{535-600}$). Protein determinations were made according to the method of Lowry et al [13]. The transport of ^{3}H-2-deoxyglucose (^{3}H-DOG, Amersham), which is non-metabolized analogue of glucose, into the rat erythrocytes was determined as in [14].

The integrity of the biomembranes in S and R rats was studied in lysosomes. Liver lysosomes were isolated as described [15] and incubated for 30 min in 0.25 M saccharose with increasing concentration of Triton X-100: 0.025, 0.05 and 0.1% of this detergent. Activities of the non-sedimentable lysosomic β-galactosidase and β-glucosidase released from lysosomes were estimated. The activity of the lysosomic enzymes were determined using 4-methylumbelliferone (4-MUF) glucosides as substrates [16]. Triton X-100 was from Serva and 4-MUF-glucopyranoside and 4-MUF-galactopyranoside supplied by Sigma. The activity of α_1-antitrypsin in rat blood serum was determined as in [17] and expressed in international units (IU) per ml.

Rearrangements of DNA in S and R rats were studied by Southern blotting with $(CAC)_5$ fingerprint probes according to [18]. Rat liver DNA was digested with BsuRI, BamH1 or HindIII restriction endonucleases, electrophoresed and hybridized with the biotin-labelled probe under standard conditions. Morphologic alterations in S rat in comparison to R rat tissues were studied as described [17].

RESULTS AND DISCUSSION

As shown in Table 1, generation of OH-radicals is about 60% higher in the liver homogenates of 2-3 month old S rats than in those of the R rats, while the difference is approximately 40% in their mitochondria. The difference is even greater between 10-12 month old S and R rats: the ·OH generation is twice higher in the liver homogenate of the S than in those of the R rats. There is no difference in ·OH generation between the myocardiums of the young S and R rats, but the difference is significant in 10-12 months old rats.

Table 1. Generation of OH-radicals in liver and myocardium of the S rats compared to that of the R rats determined by DMPO (in %)

Age:	2-3 months		10-12 months	
	homogenate	mitochondria	homogenate	mitochondria
Liver	160±10 (8)	140±9 (9)	200±15 (6)	160±10 (9)
Myocardium	105±8 (8)	103±8 (8)	160±10 (8)	140±9 (8)

The average amplitude of the SPR spectra signals of the spin adduct from the R rat samples is taken for 100%. The number of experiments is given in parentheses.

Table 2. Lipid peroxidation in the S and R rat mitochondria

Age:	2-3 months		10-12 months	
	R	S	R	S
Liver	0.61±0.11 (8)	2.12±0.31 (8)	5.34±0.63 (12)	3.72±0.71 (12)
Myocardium	0.76±0.08 (8)	1.37±0.11 (8)	0.66±0.08 (12)	0.28±0.05 (12)

Content of thiobarbiturate-reactive products in the mitochondria from the R and S rat tissue in μmoles/mg protein. The number of experiments is given in parentheses.

Table 2 demonstrates that the difference in lipid peroxidation in the mitochondria of S and R young rats is even higher than that in OH-radicals generation: it is ~3.5 times higher in the liver and ~1.8 times higher in the myocardial mitochondria of S rats. However, in 10-12 month old rats the pattern is reverse: mitochondria lipid peroxidation is higher in the R than in the S rats. The reversion has been described in the literature [19]. It has been explained by the intense exhaustion of targets for lipid peroxidation which are mainly the unsaturated fatty acids of the membrane phospholipids and lipoproteins.

We have found that the transport of monosaccharides into the S rat cells is substantially enhanced [6]. Table 3 demonstrates the intense transport of ^{3}H-2-deoxyglucose into the the S rat erythrocytes. There are data showing that autooxidation of the abundant monosaccharides results in generation of free radicals [7-9]. It was reasonable to assume that intense generation of OH-radicals in the S rat tissues may be due to the enhanced accumulation of monosaccharides in the S rat cells subjected further to oxidation and autooxidation. Enhanced generation of OH-radicals leads to a high level of lipid peroxidation. This is supported by experimental data. Feeding of S rats with galactose-rich diet containing 40% galactose for 1 week resulted in significant increase in lipid peroxidation: from 1.25±0.08 to 3.05±0.11 μmoles/mg protein of TBA-reactive products in myocardium mitochondria (as in other tissues).

Table 3. ^{3}H-DOG transport into the erythrocytes of the S and R rats (Bq×100/ml)

S	R
240±30 (6)	74±8 (6)

The number of experiments is given in parentheses.

It is known that lipid peroxidation damages biomembranes and leads to their fragility. We studied the integrity of biomembranes in S and R rats using lysosomes for monitoring. Fig.1 demonstrates that the increase in Triton X-100 concentration results in a rise in the activities of membrane-penetrating non-sedimentable lysosomic enzymes in the medium which are substantially higher in S rats than in R rats. This is indicative of enhanced fragility of biomembranes.

One of the important targets for the OH-radicals are DNA molecules where a number of modified purines and pyrimidines arise [2]. Intense modification of DNA may lead to double-strand breaks and thereby to genomic rearrangements [20].

Using as a probe oligodeoxynucleotide $(CAC)_5$ which is one of the highly repetitive sequences, we have found that in S rats there is a high rate of genomic rearrangements while R rats genomes are homologous in this respect (Fig.2). Numerous genomic rearrangements take place in S rats in spite of close inbreeding of the animals for many generations and high homozygosity. It means, obviously, that frequent DNA restructuring occurs in S rats due to the permanently acting mutagenic factors which are obviously the intensely produced endogenous oxidants.

We have found that in S rats the activity of α_1-antitrypsin in blood serum is much lower than in R rats (9.4±0.9 and 15.4±0.5 IU/ml, respectively). This may be due to intense oxidation of the methionine residues in this protein. The decrease of the α_1-antitrypsin activity leads obviously to emphyzema which is revealed by morphological studies in S rats, not in R rats.

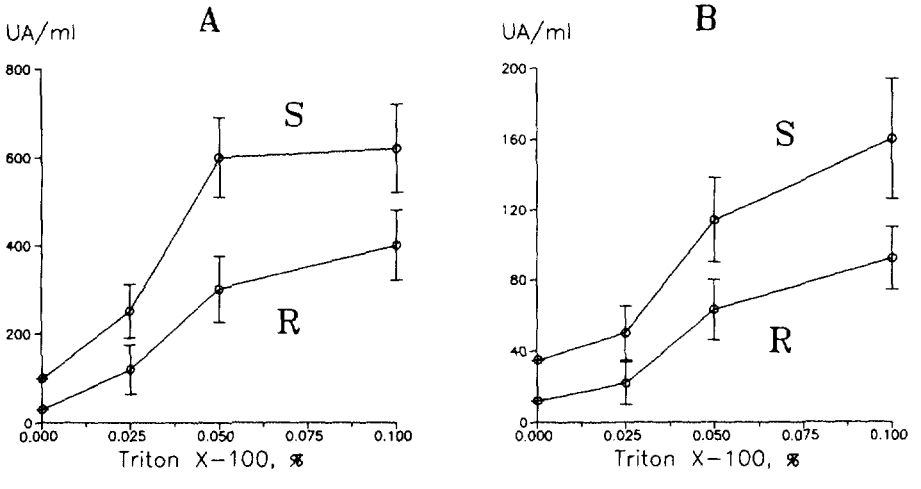
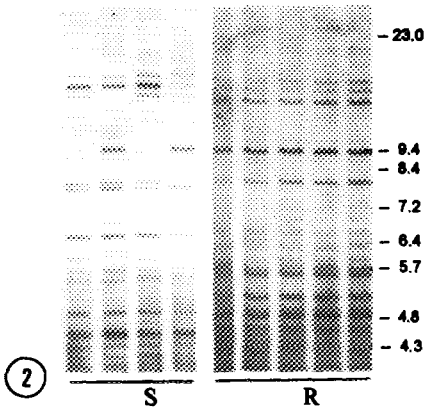


Fig.1. Fragility of the lysosomic membranes in the S and R rats tested by incubation with Triton X-100. Non-sedimented β-galactosidase (A) and β-glucosidase (B) activities were estimated. The values are means±SEM (n=6). There is a statistically significant difference between S and R rats.



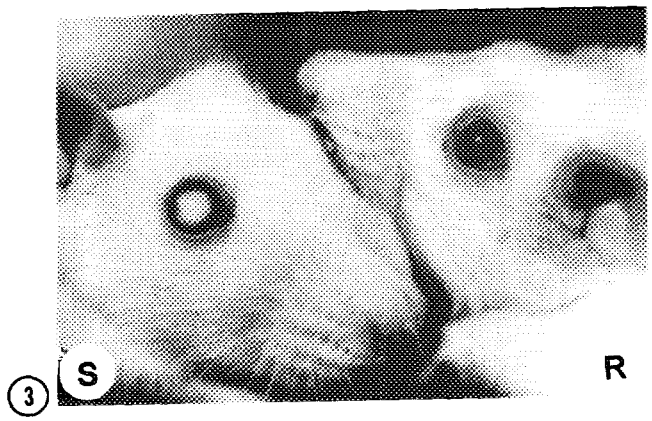


Fig.2. DNA fingerprints of the S and R rats DNA with $(CAC)_5$ probe. Rat DNA samples were digested with BsuR1, electrophoresed and hybridized with $(CAC)_5$.

Fig.3. Typical cataract in the S rat. For comparison a normal eye of the R rat is presented.

Cataracts and other lens lesions are found in ~92% of S rats (versus ~15% in R rats). They arise, obviously, as a result of the oxidative damage of the lens proteins. A typical S rat cataract is shown in Fig.3. Precataracts are observed under ophtalmoscopy as lens vesicles and multi-colored specks.

Oxidative damage to biomembranes and proteins is perhaps the cause of the myocardial alterations in the S rats resembling human hypertrophic cardiomyopathy. The mass of the hearts in S rats is higher than in R rats. Dilatation of the cardiac ventricles, hypertrophy of muscle fibers, diffuse collagenisation and sclerotic foci were observed in S rat myocardium [17].

The oxidative damage of the contractile muscles is responsible, perhaps, for the rentgenologically detected spinal column deformations found in most of the S rats which remind human congenital scoliosis (Fig.4). Due to the spine deformations the vertebrate bodies become wedge-shaped and pulpar nuclei shiffed to the deviation arc.

The numerous developmental defects were found in S rats such as inherited abnormalities of the bronchi, defects of gastric gland formation leading to polycystosis and microphtalmus among others. Benign tumors originating from the sudoral and sebaceous glands and malignant tumors of stomach, esophagus and lungs were found in many of the S rats.

Because of high embryonic mortality, fertility was lower in S than in R rats (Table 4). Analysis of the capacity to the retention of acquired task demonstrated an impaired mechanisms of long-term memory in S rats [21].

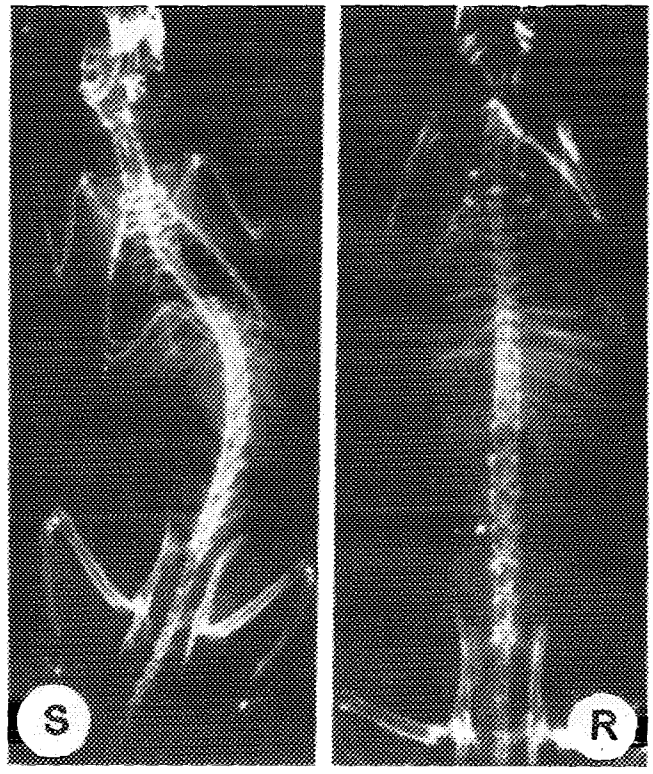


Fig.4. Spinal column deformation in the S rat revealed by roentgenography. For comparison the roentgenogram of a healthy R rat is given.

As a result of degenerative diseases, life-span was more than twice shorter in S as compared to R rats (Table 4). The fertility and longevity of R rats were close to those of Wistar rats.

It is reasonable to conclude that continuous oxidative damage results ultimately in premature aging and in early death of S rats. The damaging effect of

Table 4. Life-span and fecundity of the S rats compared to the R rats

	S	R
Life span, years	1.3-1.6 (105)	2.5-3.2 (130)
Fecundity offspring per female	4-6 (100)	8-12 (120)

The number of experiments is given in parentheses.

the OH-radicals can be enhanced by leaking lysosomic hydrolases and by other secondary effects.

To our knowledge it is for the first time that a rat strain with an inherited hyperproduction of free radicals, increased lipid peroxidation and morbid conditions reminding human degenerative diseases of aging is developed.

We have obtained preliminary data showing that predisposition to some of the degenerative diseases of aging in humans may be concerned with an inherited hyperproduction of free radicals.

REFERENCES

1. Adelman R., Saul R.L., Ames B.N. (**1988**) Proc. Natl. Acad. Sci. U.S.A. **85**, 2706-2710.
2. Imlay J.A., Linn S. (**1988**) Science **240**, 1302-1309.
3. Ames B.N., Shigenaga M.K., Hagen T.M. (**1993**) Proc. Natl. Acad. Sci. U.S.A. **90**, 7915-7922.
4. Stadtman E.R. (**1992**) Science **257**, 1220-1224.
5. Solovyova N.A., Morozkova T.S., Salganik R.I. (**1975**) Genetika (Russ.) **11**, 63-71.
6. Solovyova N.A., Ginzburg E.Kh., Kazarinova F.S., Kandaurov V.V., Salganik R.I. (**1987**) Vopr. Med. Khimii (Russ.) **33**, 984-988.
7. Morita T., Komano T. (**1983**) Agric. Biol. Chem. **47**, 11-18.
8. Thornalley P., Wolf S., Crabbe J., Stern A. (**1984**) Bioch. Biophys. Acta **797**, 276-287.
9. Mashino T., Fridovich I. (**1981**) Arch. Bioch. Biophys. **254** 547-551
10. Schneider C.W. (**1948**) J. Biol. Chem. **176**, 259-266.
11. Buettner G.R., Oberley L.M. (**1978**) Bioch. Biophys. Res. Commun. **33**, 69-74.
12. Bird R.P., Hung S.S., Hadley M., Draper H.H. (**1983**) Anal. Biochem. **128**, 240-245.
13. Lowry O.H., Rosebraugh N.J., Farr A., Randall R. (**1951**) J. Biol. Chem. **193**, 265-275.
14. Okuno I., Plesner I., Larsen T., Gliemann G. (**1986**) FEBS Lett. **195**, 303-308.
15. De Duve C., Pressman B.S., Gianette R., Wattiaux R., Appelman F. (**1955**) Biochem. J. **60**, 604-618.
16. Kolodny E.H., Muniford R.A. (**1976**) Clin. Chim. Acta **70**, 247-257.
17. Grishaeva O.N., Solovyova N.A., Semenova L.A., Semenov D.E., Salganik R.I. (**1993**) Vopr. Med. Khimii, in press.
18. Nurnberg P., Roewer L., Neitzel H., Sperling K., Popperl A., Hundrieser J., Poche H., Epplen C., Zischler H., Epplen J.T. (**1983**) Hum. Genet. **84**, 75-78.
19. Baraboy V.E., Orel V.E., Carhaul I.M. (**1991**) Lipid peroxidation and radiation, Kiev, Naukova dumka.
20. Salganik R.I., Dianov G.L. (**1992**) Mutation Res. **266**, 163-170.
21. Eliseeva A.G., Solovyova N.A., Morozkova T.S. (**1975**) Genetika (Russ.) **11**, 72-79.