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IMPAIRMENT OF RESPIRATORY FUNCTIONS IN MITOCHONDRIA OF RATS WITH AN INHERITED HYPERPRODUCTION OF FREE RADICALS

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Summary: The functional characteristics of liver mitochondria and physical-chemical properties of mitochondrial membranes were studied in S rats with congenitally enhanced capacity to free radical generation in comparison to those in Wistar rats. It was shown previously that intense lipid peroxidation, numerous DNA rearrangements, protein oxydation, morbid states resembling human degenerative diseases and short life-span are characteristic of S rats. In present study we have demonstrated that in S rats at the age of 2-3 months the respiratory rate of the mitochondria in active metabolic state is lower than in Wistar rats, and so are the values for the respiratory control ratio, oxidative phosphorylation and membrane potential. By 10-12 months of age the decrease of the respiration rate and oxidative phosphorylation in S rat mitochondria become even more dramatic. These changes are associated with a decrease in the extent of dip of proteins into the membrane lipid layer or with the increase in the amount of protein aggregates. The results add to the understanding of the nature of morbid conditions developed under the effect of intensive free radical generation and to the comprehension of their role in aging.

Oxygen -derived free radicals are widely postulated to play a casual role in cell injury, degenerative diseases and aging(1-8). Peroxidation of lipids in membranes and lipoproteins impairing their functions, oxidation of proteins leading to appearance of protein carbonyl groups and to inactivation of a number of enzymes,oxidative modification of DNA, resulting in mutations, have been suggested to be the crucial factors in cell damage caused by the oxidative stress.

Our previous studies resulted in development of S rat strain with an inherited hyperproduction of free oxygen radicals. The approaches used to develop this strain were described earlier (9-12). The S rats show intense peroxidation of lipids, numerous DNA rearrangements (11-12), high level of protein oxidation (13). Morbid conditions resembling human degenerative diseases: cataracts, emphysema, scolyosis, cardiovascular lesions, tumours are characteristic of these animals. Short life-span and premature aging are their salient features.

Mitochondria are active in continuous generation of semiquinone radicals, reactive oxygen species and in lipid peroxidation (14-15). Their respiratory functions decline with age presumably due to the long-lasting generation of free radicals resulting in accumulation of lesions incured to the mitochondrial genome and to other mitochondria structures (16-17).

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We showed previously that generation of OH-radicals and the level of lipid peroxidation in the mitochondria of S rats are substantially higher than in the controls (11-12).

It was reasonable to suggest that lesions of mitochondrial structures under conditions of continuous oxidative stress may result in a heavy impairment of mitochondrial functions.

In this study we analyse the respiratory functions and membrane peoperties of liver mitochondria from S rats in comparison to those from Wistar rats. Deterioration of the S rat mitochondria structures and functions are described.

MATERIALS AND METHODS

This study was performed on S rats at the age of 2-3 months and 10-12 months. Wistar rats of these age served as controls. To develop the S rat strain the rats of the Wistar strain were selected for high sensitivity to the cataractogenic effect of galactose-rich diet , and were maintained further by strict and close inbreeding for more than 40 generations (9-12).

The mitochondria were isolated from the S- and Wistar rat livers according to the method of Vercesi et al. (18) with slight modification. Differential centrifugation was performed in a buffer containing 250 mM sucrose, 1 mM EDTA, 10 mM Tris-HCl (pH 7.4). Repeated centrifugation of the mitochondria was carried out in a buffer of the same composition but without EDTA . The final precipitate of the mitochondria was resuspended to a concentration of 60-80 mg of protein per ml of the medium. The concentration of mitochondrial protein was determined according to Lowry et al. (19). Respiratory rate , ADP/O ratio and phosphorylation activity of the mitochondria were estimated as described (20). The succinate and glutamate-malate supported respiration rates of the S and Wistar rat liver mitochondria were determined polarographically in a termostated cuvette equipped with a Clark oxygen electrode at 25°C according to Easterbrook (20). The incubation medium contained 125 mM KCl, 20 mM Tris-HCl (pH 7.4), 5mM KH_2PO_4 and 1 mM $MgCl_2$. As oxidative substrate 10 mM succinate with 2 mg/ml of rotenone (to inhibit NAD-linked substrate oxidation) or 10mM glutamate plus 2 mM malate were added to the incubation medium. The addition of succinate or glutamate plus malate induced a slow oxygen uptake (state 4) while the addition of ADP (150 mM) stimulated the respiration to the active state (state 3). The respiratory control ratio (RCR) was measured as quotient of the respiratory rate of state 3 to that of the state 4. The oxydation phosphorylation activity was expressed as the ADP/O ratio which is the quotient between the amount of ADP (in nmol) added and the oxygen (in ng-atom) consumed during the state 3. To measure the mitochondrial respiratory rate in an uncoupled state, 0.5 mM of carbonyl cyanide m-chlorophenyl hydrazone (Sigma) as an uncoupling agent (21) was added. Measurements of the membrane potential were performed with the use of 4-N-(dimethylaminostiryl)-1-methylpyridinium as a fluorescent probe (Zonde, Latvia) in a Hitachi MPF-4 spectrofluorimeter as described (22). The viscosity of mitochondrial membranes was evaluated also with the use of the fluorescent probe (22,23). The measurements of the efficiency of the fluorescence energy transfer from the tryptophan residues of the mitochondrial membrane proteins to the pyrenes within the lipid membrane layer allowed to evaluate the interaction of the membrane proteins and lipids, the changes in the dip of proteins into the membrane lipids as a percentage of proteins not contacting with the lipids (22). The osmotic properties of mitochondria were evaluated by the capacity of hypertonic sucrose solution to produce mitochondria contraction(28). The latter were registered by the alteration of mitochondria optical properties in a Hitachi-556 spectrofluorimeter at 546 nm: 0.5 mg of mitochondria were incubated for 10 min at 37°C in 3 ml cuvette in

a buffer containing 5 mM Tris-HCl (pH 7.4), 125 M KCl, 1 mg rotenone / mg protein where 100 Ml of 2 M sucrose were added.

RESULTS AND DISCUSSION

Liver mitochondrial respiratory functions were studied in S and Wistar rats aged 2-3 and 10-12 months. The respiration supporting substrates ,succinate or glutamate plus malate, develop reducing equivalents at different mitochondria sites (complex I and complex II ,accordingly). We have found substantially lower respiratory activities in state 3 and in uncoupled state in S rat mitochondria in comparison with those in Wistar rats (Table 1). Respiratory control ratio was also considerably lower in S rats.

The decrease of oxidation rate of the substrates in active metabolic state produced a dramatic fall in the phosphorylation activity of the mitochondria (Table 2). Inhibition

Table 1. Liver mitochondria respiratory functions in S and Wistar (W) rats of different age

	Rate of respiration (ng-atom 0 / min/mg protein)			
	Rat age (months)			
	2-3		10-12	
Mitochondria state	Rat strain			
	W	S	W	S
	Supported by succinate			
State 3	127.1+ 6.5	81.1 + 8.6	114.5 + 9.9	66.3 + 6.9*
State 4	28.6 + 2.0	23.0 + 1.7	25.6 + 1.5	25.6 + 1.9
Uncoupled state	187.1 + 10.8	165.2 + 12.9	176.5 + 14.8	128.7 + 11.7*
RCR	4.34 + 0.22	3.63 + 0.11*	4.48 + 0.2	2.65 + 0.24 **
	Supported by glutamate & malate			
State 3	70.3 + 2.1	49.9 + 3.5	69.9 + 9.5	43.4 + 6.1
State 4	17.4 + o.9	13.2 + o.9	13.5 + 0.6	14.5 + 0.7
Uncoupled state	87.4 + 2.7	66.2 + 4.9*	96.7 + 9.1	59.8 + 7.1*
RCR	5.o + 0.33	3.83 + 0.12*	5.34 + o.47	2.98 + 0.35**

Values shown are the means+ SE for the number of studied mitochondria preparations (n=5) each from the individual animal.Differences for respiratory parameters for W and S rats are significant at P< 0.01(*) or at P< O.O5(**).

Table 2. Liver mitochondria phosphorylation activity in S and Wistar (W) rats of different age

Phosphorylation parameters	Rat age (months)			
	2-3		10-12	
	Rat strain			
	W	S	W	S
	Supported by succinate			
ADP/O	1.78 + 0.05	1.79 + 0.06	1.73 + 0.05	1.52 +0.04**
Phosphorylation rate	225.0 + 10.0	157.1 + 9.5*	193.7 + 17.3	102.9 ≠ 12.7**
	Supported by glutamate & malate			
ADP/O	2.68 + 0.06	2.55 + 0.06	2.51 + 0.10	2.10 + 0.09
Phosphorylation rate	183 + 8.8	131.2 + 9.7*	166.1 + 1.3	92.6 + 10.4**

Values shown are the means ± SE for the number of studied mitochondria preparations (n=5) each isolated from the individual animal. Differences between phosphorylation parameters for W and S rats are significant at $P < 0.01$(*) or at $P < 0.05$(**).

of the respiratory chain of mitochondria, in turn, led to a decrease in the value of the membrane pontential (Table 3). The data presented clearly show that the decrease in respiratory functions in older S rats is much more pronounced than in young ones.

It is known that the activities of NADH-dehydrogenase, NADH-oxidase, succinate oxidase and F_0-F_1-ATPase are inhibited by superoxide radicals and hydroperoxides (24,25). This effect of free radicals is regarded as a result of modification of amino acid residues, preferentially of tyrosine, phenylalanine,triptophane and histidine which under protein oxidation acquire carbonyl groups while SH-groups of methionine ans cysteine residues are also oxidized(6-8). Intensive oxidation of proteins was found in S rats (13). It was shown previously that the fragility of cell membranes is characteristic of S rats obviously as a result of high levels of lipid peroxidation and protein oxidation (12). Modification of mitochondrial DNA under the effect of free radicals is responsible for the structural mutations (17,26,27). It is pertinent to recall that numerous DNA rearrangements occur in S rats in spite of close inbreeding they have been subjected to for many generations (12). It is reasonable to assume that heavy impairment of mitochondrial respiratory functions in S rats is due to the proteins, lipids and DNA lesions which occur, apparently , under the effect of intensive continuous radical generation.

The lesions accumulate with the age and result in progressive deterioration of the mitochondrial functions.

Table 3. Characteristics of liver mitochondrial membranes in S and Wistar (W) rats of different ages

Membrane parameters	Rat age (months)			
	2-3		10-12	
	Rat strain			
	W	S	W	S
Lipid viscosity (puas)	0.99 + 0.17	1.04 + 0.08	1.43 + 0.9	1.28 + 0.11
Protein-lipid interaction(%)	46.1 + 1.2	43.1 + 1.3	47.2 + 0.9	52.2 + 1.2**
Membrane potential mV	200.7 + 5.3	183.6 + 3.9*	192.1 + 4.6	177.3 + 2.9**

Values shown are the means + SE for the number of studied mitocnondria preparations (n=5) each isolated from the individual animal. Differences between mitochondrial membrane preparations are significant at $P<0.01$(*) or at $P<0.05$(**).

Our data on the properties of mitochondria, in particular, of those related to the capacity of a hypertonic sucrose solution to produce to the same extent the contraction of mitochondria in the S and the control rats evidence that the inner mitochondrial membranes of the S rat liver lacks nonspecific permeability.

Studies of the structure of mitichondrial membranes with the use of fluorescent probes revealed no significant differences in the viscosity of the membrane lipids between the Wistar and the S rats. However, a noteworthy feature is the age-related trend in increase in lipid viscosity in both Wistar and S rats (Table 3).

Evaluation of the efficiency of fluorescence energy transfer from tryptophan residues of mitochondrial proteins to pyrenes located in the membrane lipids gives an idea of the state of protein-lipid interaction in the mitochondrial membranes. The data of Table 3 show that in young S rats there is a tendency to a decrease of the percentage of proteins not contacting with lipids, i.e. those proteins which are at a distance exceeding the critical one from lipids (the distance has been estimated as 3.6 nm for a tryptophan-pyrene pair). By the age of 10-12 months this distance increases greatly, thereby indicating a decrease in a dip of proteins or/and an increase in the amount of associated proteins in the mitochondrial membranes of the S rats. Changes in the protein-lipid interaction in the S rat mitochondria are consistent with the possibility of formation of cross-linked protein aggregates under the effct of free radicals (28).

Thus, these studies of the functional state of the liver mitochondria and the physical and chemical characteristics of their membranes in the S rats with enhanced capacity for radical generation revealed the impairment of respiratory rates and oxidative phosphorylation as well as deterioration of the integrity of the mitochondrial membranes.

The damage to the mitochondrial genome and obviously to other mitochondria structures caused by free radicals is regarded as a major contributory factor to aging (16,17) and to a number of diseases (29). From this point of view the S rat strain seems to be a very promising model for continuing studies in our quest for free radical-mediated degenerative diseases and aging.

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