Generation of Reactive Oxygen Species by Mitochondria in Senescence-Accelerated OXYS Rats

E. B. Men'shchikova, I. G. Shabalina*, N. K. Zenkov, and N. G. Kolosova**

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 133, No. 2, pp. 207-210, February, 2002
Original article submitted October 23, 2001

Chemiluminescence assay showed that oxygen reduction and production of superoxide anion and hydrogen peroxide by liver mitochondria in OXYS rats highly sensitive to oxidative stress were less intensive than in Wistar rats. Experiments with cytochrome *c* oxidase inhibitors showed that decreased $O_2^{\bullet-}$ generation in mitochondria of OXYS rats is probably associated with changes in complex III of the electron transport chain.

Key Words: *OXYS rats; reactive oxygen species; mitochondria*

Oxidative stress and disturbances in ion homeostasis and energy metabolism play the major role in the pathogenesis of age-related pathologies and aging [12]. These processes are closely associated with activity of mitochondria, the main source of endogenous prooxidants in cells. Mitochondria are highly sensitive to oxidative stress, which can be explained by low content of protective histones and low activity of DNA-repairing systems in mitochondrial genome. R. A. Floyd *et al.* [9] reported that mitochondria are the Achilles' heel in aging. OXYS rats were obtained by selection and inbreeding of Wistar rats sensitive to the cataractogenic effect of galactose (Institute of Cytology and Genetics). Previous studies showed that structural and functional disturbances in mitochondria underlie accelerated aging in OXYS rats [1]. The maximum life span of OXYS rats is 28% lower than that of Wistar rats. These animals are characterized by early cataract formation, involutional changes in internal organs, and disorders of higher nervous activity typical of aging humans and animals [3-5,15]. The severity of pathological changes in mitochondria observed in young OXYS rats increases during aging. Functional disturbances include low phosphorylation rate during oxidation of glutamate, malate, and succinate. Biochemical changes are manifested in a decrease in cytochrome *a* content and F_1F_0-ATP-synthetase activity and increase in cytochrome b_5 concentration. Ultrastructural changes include a decrease in mitochondrial volume and surface densities and appearance of mitochondria with destructed cristae and lysed matrix [1,4]. These data suggest that mitochondria of OXYS rats intensively generate oxygen radicals detected in tissues by electron paramagnetic resonance [15]. It should be emphasized that this is a special characteristics of OXYS rats [14]. Here we studied generation of reactive oxygen species (ROS) by mitochondria in Wistar and OXYS rats of different age by recording chemiluminescence (CL) with various luminophores.

MATERIALS AND METHODS

We used sucrose, D,L-glutamic acid, sodium salts of malate, succinate, and pyruvate, Tris-HCl, bovine serum albumin (BSA), rotenone, KCN, ethylene glycol-bis(b-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), horseradish peroxidase, luminol, lucigenin (Sigma), zymosan (Olain Plant of Medicinal Preparations), KCl, KH_2PO_4, and $MgCl_2$.

Research Center for Clinical and Experimental Medicine, Siberian Division of the Russian Academy of Medical Sciences; *Institute of Biochemistry, Siberian Division of the Russian Academy of Medical Sciences; **Institute of Cytology and Genetics, Siberian Division of the Russian Academy of Sciences, Novosibirsk. ***Address for correspondence:*** lemen@online.sinor.ru. Men'shchikova E. B.

0007-4888/02/1332-0175$27.00

Experiments were performed on 20 male OXYS rats aging 3-4 months (190-220 g) and 15 male OXYS rats aging 12-13 months (300-330 g). Wistar rats of the same age weighing 200-220 and 300-360 g, respectively, served as the control. The animals were obtained from the Institute of Cytology and Genetics and kept under standard conditions. Liver mitochondria were isolated by differential centrifugation in the medium containing 250 mM sucrose, 1 mM EGTA, 0.1% BSA, and 10 mM Tris-HCl (pH 7.4). The homogenate was centrifuged at 700*g* for 10 min. Mitochondria were precipitated by centrifugation at 10,000*g* for 10 min, resuspended in a EGTA free medium, and centrifuged at 11,000*g* for 10 min. These procedures were performed at 0-3°C. The precipitate was resuspended in the EGTA free medium (40-60 mg protein/ml medium). Protein content was measured by the method of Lowry using BSA as the standard.

CL of mitochondria with luminophores was measured on a Foton chemiluminometer [11]. The reaction mixture contained 1 mg mitochondrial protein in 1 ml incubation medium (100 mM sucrose, 50 mM KCl, 20 mM Tris-HCl, 5 mM KH_2PO_4, 1 mM $MgCl_2$, and 1 mM EGTA; pH 7.4). Pyruvate (10 mM) and malate (2 mM), glutamate (10 mM) and malate (2 mM), and succinate (10 mM) were used as the oxidation substrates. To evaluate intramitochondrial $O_2^{\bullet-}$ production we recorded CL after the addition of 20 μM lucigenin into a cuvette in the absence or presence of 2 mM KCN or 1 μM rotenone. $O_2^{\bullet-}$ production by mitochondria was estimated by the average intensity of CL from the 7th to 10th minute of recording. In this period spontaneous CL of mitochondria reached a plateau and remained unchanged. H_2O_2 production was estimated by the intensity of CL in the presence of 50 μM luminol and 50 μg/ml horseradish peroxidase.

RESULTS

We found a linear correlation between CL of rat liver mitochondria and lucigenin concentration (Fig. 1). Lucigenin in a concentration of more than 20 μM undergoes oxidation-reduction transformations, which intensifies $O_2^{\bullet-}$ generation [11]. For a comparative study we used lucigenin in a concentration of 20 μM.

In OXYS and Wistar rats the intensity of intramitochondrial $O_2^{\bullet-}$ generation estimated by lucigenin-induced CL decreased with aging ($p<0.1$, Table 1). Similar age-related changes were observed after the addition of respiratory inhibitors KCN and rotenone to mitochondria. However, in young and old OXYS rats the intensity of $O_2^{\bullet-}$ production was lower than in Wistar rat ($p<0.1$, Table 1). The inhibition of complex IV in the electron transport chain with KCN intensified intramitochondrial $O_2^{\bullet-}$ generation in young and old Wistar rats by 48% ($p<0.001$). However, in young and old OXYS rats the intensity of $O_2^{\bullet-}$ production increased only by 20% ($p<0.05$ compared to the corresponding parameter without KCN) and 10% (insignificant), respectively.

Recording of luminol-induced CL after the addition of horseradish peroxidase in low concentrations is a sensitive method for detecting H_2O_2 in biological systems [10]. Changes in the intensity of luminol-induced mitochondrial CL in the presence of horseradish peroxidase and lucigenin-induced CL were similar. CL of mitochondria markedly decreased in OXYS rats (Table 1).

In OXYS rats the intensity of lucigenin-induced CL of isolated mitochondria reflecting $O_2^{\bullet-}$ production [11] was much lower than in Wistar rats (Table 1). The intensity of lucigenin-induced CL in OXYS rats decreased in incubation media with various oxidation substrates. These results indicate that the impairment of intramitochondrial $O_2^{\bullet-}$ production is not associated with changes in complexes I and II of the electron transport chain. Coenzyme Qa component of complex III, is the major producer of $O_2^{\bullet-}$ in mitochondria. After the inhibition of complex IV in the electron transport chain by KCN, the increase in CL of mitochondria in OXYS rats was less pronounced than in Wistar rats. Therefore, these animals are characterized by different oxidation-reduction transformations of ubiquinone.

The impairment of intramitochondrial $O_2^{\bullet-}$ production in OXYS rats may result from high activity of Mn-superoxide dismutase (Mn-SOD). We mea-

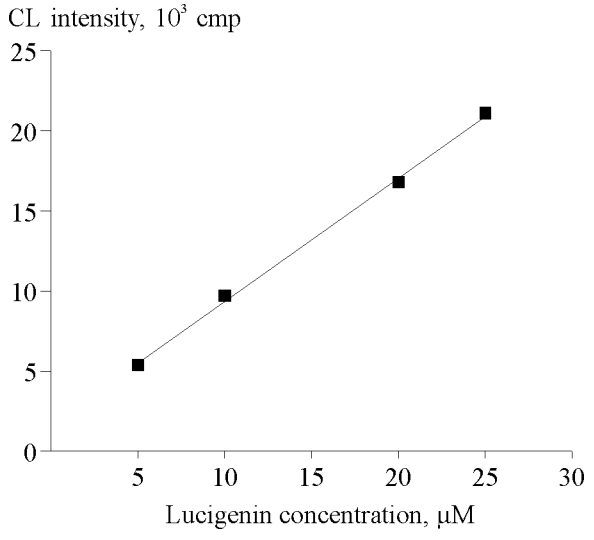

Fig. 1. Intensity of chemiluminescence (CL) of liver mitochondria from Wistar rats as a function of lucigenin concentration.

TABLE 1. CL of Liver Mitochondria from Wistar and OXYS Rats of the Same Age (10^3 cpm, $M \pm m$)

Incubation medium	3-4 months		12-13 months	
	Wistar	OXYS	Wistar	OXYS
Pyruvate and malate+lucigenin	184.1±29.9	123.3±23.9	119.3±19.4	76.3±10.6**
Pyruvate and malate+lucigenin+KCN	243.8±22.9	148.5±31.4*	164.0±25.6^{+}	84.1±14.2^{*++}
Pyruvate and malate+lucigenin+rotenone	30.9±6.1	19.5±4.6	18.0±3.2	13.4±2.4
Glutamate and malate+lucigenin	112.2±6.0	98.3±6.4	141.4±12.0^{+}	77.2±8.7^{*++}
Succinate+lucigenin	149.8±13.9	107.4±14.3**	144.9±4.4	84.7±10.8*
Succinate+luminol+horseradish peroxidase	184.0±36.3	105.0±46.7	323.5±157.6	34.1±5.5**

Note. *p<0.05 and **p<0.1 compared to Wistar rats; ^{+}p<0.05 and ^{++}p<0.1 compared to 3-4-month-old animals.

sure luminol-induced CL in the presence of horseradish peroxidase that reflects H_2O_2 production [10]. In mitochondria from OXYS rats, the intensity of H_2O_2 production decreased to a greater degree than $O_2^{\bullet-}$ generation (Table 1). Mn-SOD is an inducible enzyme, whose synthesis is initiated by superoxide anions. The impairment of $O_2^{\bullet-}$ production is followed by a decrease in the concentration of mitochondrial Mn-SOD. These changes are accompanied by the inhibition of H_2O_2 production, which is formed in the reaction of $O_2^{\bullet-}$ dismutation catalyzed by SOD.

Our results do not contradict published data that the addition of H_2O_2 intensifies $OH^{\bullet}$ production in the suspension of mitochondria [15]. If the generation of $O_2^{\bullet-}$ depends on the mitochondrial electron transport chain, H_2O_2 degradation is determined by the presence of metal ions of alterating valence and hemic cytochromes. OXYS rats have high content of cytochrome b_5 on the outer mitochondrial membrane [6]. It should be emphasized that ubiquinone possesses prooxidant (reduction of O_2 into $O_2^{\bullet-}$) and antioxidant activities (inhibition of $OH^{\bullet}$ and lipid and tocopherol radicals) [7]. The decrease in ubiquinone content suppresses $O_2^{\bullet-}$ production, but intensifies free radical oxidation of lipids.

Our results indicate that the intensity of $O_2^{\bullet-}$ and H_2O_2 production in the electron transport chain of liver mitochondria is low in OXYS rats. The state of mitochondria in OXYS rats may be characterized as "soft disintegration" (V. P. Skulachev). A slight decrease in the transmembrane electrical potential (ΔY) and intensification of non-phosphorylative respiration maintain the concentrations of O_2 and single-electron reducing agents (*e.g.*, ubisemiquinone radical $^{\bullet}$QH) at low level. This decreases the risk of $O_2^{\bullet-}$ formation [13]. Normal aging is accompanied by similar changes. Moreover, the increase in proton conductance of the inner mitochondrial membrane is considered as "uncoupling for saving" [8]. Despite insignificant production of ROS, the inhibition of oxidative phosphorylation in mitochondria of OXYS rats and age-related energy deficiency [6] shift the prooxidant-antioxidant balance toward the prevalence of prooxidants, which is followed by oxidative stress [2]. These data suggest that progressive mitochondrial dysfunction plays the major role in accelerated aging and development of oxidative stress-associated diseases in OXYS rats. There is no agreement regarding the role of ROS in biological processes. Our results and published data indicate that OXYS rats can be used for evaluation the contribution of ROS into normal and accelerated aging and for elaboration of new preventive methods.

This work was supported by the Russian Foundation for Basic Research (grant No. 99-04-49744).

REFERENCES

1. N. G. Kolosova, S. V. Aidagulova, and G. I. Nepomnyashchikh, *Byull. Eksp. Biol. Med.*, **132**, No. 8, 235-240 (2001),
2. N. G. Kolosova, N. A. Solov'yova, I. G. Shabalina, and R. I. Salganik, *Ibid.*, **121**, No. 3, 282-284 (1996).
3. L. V. Loskutova and N. G. Kolosova, *Ibid.*, **129**, No. 8, 155-158 (2000).
4. E. L. Lushnikova, L. M. Nepomnyashchikh, O. A. Postnikova, and V. G. Tsimmerman, *Ibid.*, **128**, No. 8, 233-237 (1999).
5. N. A. Solovyova, T. S. Morozkova, and R. I. Salganik, *Genetika*, **11**, No. 11, 63-71 (1975).
6. I. G. Shabalina, N. G. Kolosova, A. Yu. Grishanova, *et al.*, *Biokhimiya*, **60**, No. 12, 2045-2052 (1995).
7. R. E. Beyer, *Free Radic. Biol. Med.*, **8**, 545-565 (1990).
8. M. D. Brand, *Exp. Gerontol.*, **35**, 811-820 (2000).
9. R. A. Floyd, M. West, and K. Hensley, *Ibid.*, **36**, 619-640 (2001).
10. L. Li, M. A. Arnold, and J. S. Dordick, *Biotechnol. Bioeng.*, 41, 1112-1120 (1993).
11. Y. Li, H. Zhu, and M. A. Trush, *Biochim. Biophys. Acta*, **1428**, 1-12 (1999).
12. M. P. Mattson, *Nat. Rev. Mol. Cell. Biol.*, **1**, 120-129 (2000).
13. S. Papa and V. P. Skulachev, *Mol. Cell. Biochem.*, **174**, 305-319 (1997).
14. *Rat Genome*, **12**, 52-54 (1996).
15. R. I. Salganik, N. A. Solovyova, S. I. Dikalov, *et al.*, *Biochem. Biophys. Res. Commun.*, **199**, 726-732 (1994).