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Localization at Complex I and Mechanism of the Higher Free Radical Production of Brain Nonsynaptic Mitochondria in the Short-Lived Rat Than in the Longevous Pigeon

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Free radical production and leak of brain nonsynaptic mitochondria were higher with pyruvate/malate than with succinate in rats and pigeons. Rotenone, antimycin A, and myxothiazol maximally stimulated free radical production with pyruvate/malate but not with succinate. Simultaneous treatment with myxothiazol plus antimycin A did not decrease the stimulated rate of free radical production brought about independently by any of these two inhibitors with pyruvate/malate. Thenoyltrifluoroacetone did not increase free radical production with succinate. No free radical production was detected at Complex IV. Free radical production and leak with pyruvate/malate were higher in the rat (maximum longevity 4 years) than in the pigeon (maximum longevity 35 years). These differences between species disappeared in the presence of rotenone. The results localize the main free radical production site of nonsynaptic brain mitochondria at Complex I. They also suggest that the low free radical production of pigeon brain mitochondria is due to a low degree of reduction of Complex I in the steady state in this highly longevous species.

KEY WORDS: Aging; hydrogen peroxide; mitochondria; longevity; bird.

INTRODUCTION

More than 90% of oxygen is consumed at mitochondria. This makes these organelles the main source of oxygen radicals in tissues of healthy animals. Localization of the mitochondrial site of free radical generation is of paramount importance due to growing evidence about the implication of these highly damaging substances in aging (Harman, 1994; Ozawa, 1995) and degenerative diseases such as Parkinson's disease (Shapira, 1994), with special reference to mitochondria (Ku *et al.*, 1993; Barja *et al.*, 1994; Asunción *et al.*, 1996; Yu *et al.*, 1996; Herrero and Barja 1997a,b; see Shigenaga and Ames, 1994 and Richter, 1995 for review). Nevertheless, almost all previous studies about the site of free radical production have been conducted in heart mitochondria (Boveris *et al.*, 1976; Takeshige and Minakami, 1979; Cadenas and Boveris, 1980; Nohl and Jordan, 1986; Ambrosio *et al.*, 1993; Nohl, 1994; Herrero and Barja, 1997a; Hansford *et al.*, 1997). Few authors have approached the problem in other organs highly relevant for aging and whole-body homeostasis like the brain, and much work has been performed in rats, animals with very short life span.

The rate of succinate-supported mitochondrial free radical generation decreases as the maximum life span (MLSP)[2] of the donor species increases; this was found in various tissues from six mammals which follow the inverse relationship between the weight-

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[2] Abbreviations: MLSP, maximum life span; TTFA, thenoyltrifluoroacetone; TMPD, tetramethylphenylenediamine.

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specific basal metabolic rate and the MLSP and ranging in body size from that of rat to that of cow (Sohal *et al.*, 1989, 1990; Ku *et al.* 1993). The slow rate of mitochondrial H_2O_2 production of the species with higher body size and MLSP (like the cow) used in these studies would be due to their low basal metabolic rate and thus to their low mitochondrial oxygen consumption and electron flow (Ku *et al.*, 1993). Mitochondrial free radical generation could be the causal link for the well-known inverse relationship between the weight-specific metabolic rate and the MLSP present in many (but not all) animals (Pearl, 1928; Prothero and Jürgens, 1987). Nevertheless, this inverse relationship could be also due in principle to other unknown factors causing aging and positively associated with the specific metabolic rate. In order to clarify if the relationship between the rate of mitochondrial free radical production and MLSP is more than circumstantial it is imperative to study animal species in which the MLSP is substantially higher than expected from their metabolic rates. This is known to take place in two homeothermic animal groups: primates and birds. In these two cases, the MLSP is between two and four times greater than in the majority of mammals of similar body size or specific metabolic rate (Calder, 1985; Prothero and Jürgens, 1987; Holmes and Austad, 1995). The bird case is specially striking since they are the only Class of animals showing both a high basal specific metabolic rate and a high MLSP. In this case, it is obvious that the high MLSP is not caused by a slow metabolism; analogously, the comparatively low metabolic rate of humans is not enough to explain their extraordinarily high longevity. The high basal oxygen consumption of birds would constitute a problem for the free radical theory of aging only if it necessarily leads to a high rate of free radical generation.

But recent data from our laboratory have shown that succinate-supported H_2O_2 production of crude brain mitochondria is lower in pigeons than in rats (Barja *et al*, 1994). Body size and basal weight-specific metabolic rate are similar in both species but rats have a MLSP of 4 years, whereas pigeons show, like birds in general, an extraordinarily high longevity (MLSP = 35 years in the pigeon) of unknown origin. All the information available to date shows that a low mitochondrial oxygen radical production is a characteristic of longevous species (large mammals, or birds), no matter if they have low or high basal metabolic rates. Nevertheless, in the majority of previous investigations concerning mitochondrial free radical production and MLSP in mammals (Ku *et al.*, 1993; Sohal *et al.*, 1989, 1990) or birds (Barja *et al.*, 1994) only a Complex II-linked substrate (succinate), which bypasses Complex I, was used, and the effects of specific inhibitors of the respiratory chain inhibitors were not studied.

Even though a low mitochondrial free radical production has been found previously in longevous species, neither the mechanisms which allow it or the mitochondrial sites where this occurs have been previously investigated. In the present paper, the rate and site of free radical production is studied in brain free (nonsynaptic) mitochondria of rats and pigeons purified with Ficoll gradients, using various substrates and inhibitors specific for different segments of the respiratory chain. The main free radical generation site is exclusively localized, in this particular kind of mitochondria, at Complex I. The study also shows a mechanism which allows brain mitochondria of the longevous species to generate oxygen reactive metabolites at a low rate without the need of a decrease in oxygen consumption which could compromise useful energy production.

MATERIALS AND METHODS

Preparation of Nonsynaptic Free Mitochondria

Animals were acclimated at the laboratory during at least one week to 25°C and 12:12 L:D illumination conditions. Young mature adult male Wistar rats (Iffa-Creddo, Lyon, France) had 4–6 months of age and mature adult male pigeons (*Columba livia*; obtained from a spanish breeder) had 2–4 years of age. These age ranges were selected to obtain young adults within 6–12% of the MLSP of each species. The animals were sacrificed by decapitation and the brains were rinsed several times, chopped, and manually homogenized with a loose fitting pestle in 35 ml of isolation medium (250 mM sucrose, 0.5 mM K^+-EDTA, 10 mM Tris-HCl, pH 7.4). The homogenate was centrifuged at 2,000*g* for 3 min and this centrifugation protocol was repeated in the first supernatant. The second supernatant was centrifuged at 12,500*g* for 8 min to obtain the crude mitochondrial pellet. Nonsynaptic mitochondria were obtained from this pellet by centrifugation in Ficoll gradients according to the procedure of Lai and Clark (1979) with small modifications.

Mitochondrial Oxygen Radical Production

The H_2O_2 production of brain mitochondria was measured by a kinetic method (Barja *et al.*, 1994; Barja, 1998) following the linear increase in fluorescence (excitation at 312 nm, emission at 420 nm) due to oxidation of homovanillic acid by H_2O_2 in the presence of horseradish peroxidase in an LS50B computer-controlled Perkin-Elmer fluorometer. This method is at the same time specific, more sensitive than many previously used spectrophotometric H_2O_2 detection methods, and free from the interference of mitochondrial antioxidants, and the molecular H_2O_2 detector probes used do not alter the respiratory control index (Barja, 1998). Its almost instantaneous response to H_2O_2 makes it appropriate to study the effects of specific respiratory inhibitors on the rate of mitochondrial oxygen radical generation.

Reaction conditions were 0.4 mg of mitochondrial protein per ml (both in rat and pigeon mitochondria), 6 U/ml of horseradish peroxidase, 0.1 mM homovanillic acid, 50 U/ml of superoxide dismutase, and 10 mM succinate, 5 mM pyruvate/2.5 mM malate, or ferrocytochrome *c* (24 μM), added at the end to start the reaction in the same incubation buffer used for the oxygen consumption measurements (145 mM KCl, 30 mM Hepes, 5 mM KH_2PO_4, 3 mM $MgCl_2$, 0.1 mM EGTA, pH 7.4, at 30°C). The experiments with ferrocytochrome *c* were performed in the presence of 10 μM myxothiazol to prevent electron flow through Complex III and were continued during at least 10 min of incubation in hypotonic buffer (30 mM Hepes, 5 mM KH_2PO_4, 3 mM $MgCl_2$, 0.1 mM EGTA, pH 7.4). This procedure opens the outer mitochondrial membrane allowing the ferrocytochrome *c* to reach the respiratory chain (Sherratt *et al.*, 1988). Since the superoxide dismutase added in excess converts all O_2^- produced (if any) to H_2O_2, the measurements estimate the total (O_2^- plus H_2O_2) mitochondrial production of reactive oxygen species. Conversion of fluorescence units to nanomoles of H_2O_2 was performed with a standard curve using glucose plus glucose oxidase as H_2O_2 generator and the results were expressed as nanomoles of H_2O_2 produced per min per mg protein. The cuvette medium was constantly stirred with a built-in electromagnetic stirrer (biokinetic accessory of the LS50B) and bar flea. This allowed the rapid mixing of the inhibitors, which were added during each kinetic experiment by slightly opening the sample compartment of the fluorometer.

Mitochondrial Oxygen Consumption

The oxygen consumption of brain nonsynaptic mitochondria was measured at 30°C in a water-thermostatted incubation chamber with a Clark-type O_2 electrode and O_2 control box (CB1-D Hansatech) in 0.5 ml of incubation buffer (145 mM KCl, 30 mM Hepes, 5 mM KH_2PO_4, 3 mM $MgCl_2$, 0.1 mM EGTA, pH 7.4) with 0.4 mg of mitochondrial protein per ml and 10 mM succinate or 5 mM pyruvate/2.5 mM malate as substrates, in the absence (State 4) and in the presence (State 3) of 500 μM ADP. Brain mitochondria from rats and pigeons routinely had a mitochondrial respiratory control ratio (State 3/State 4 oxygen consumption) around 5.0 with pyruvate/malate. The experiments with ferrocytochrome *c* (24 μM) as substrate were performed in the presence of 10 μM myxothiazol (to prevent electron flow trough Complex III) and in hypotonic buffer (30 mM Hepes, 5 mM KH_2PO_4, 3 mM $MgCl_2$, 0.1 mM EGTA, pH 7.4; in order to allow ferrocytochrome *c* to reach the inner mitochondrial membrane, Sherratt *et al.*, 1988).

Mitochondrial Inhibitors

The concentrations of all respiratory inhibitors used were the same in the free radical production and oxygen consumption measurements. Previous studies from our laboratory (Herrero and Barja, 1997a) using inhibitors specific for different segments of the respiratory chain (Fig. 1) have found that 2 μM rotenone, 10 μM antimycin A, 10 μM TTFA, and 10 μM myxothiazol totally abolish the respiratory control ratio without affecting (rotenone, TTFA, and myxothiazol) or minimally depressing (by 6% with antimycin A) the chemical H_2O_2 detection system (horseradish peroxidase and homovanillic acid) in relation to their effects on the rate of free radical production of pyruvate/malate-supplemented brain mitochondria (90% stimulation with antimycin A).

Mitochondrial Free Radical Leak

The simultaneous measurement in parallel of H_2O_2 production and oxygen consumption using the same buffer, temperature, and concentrations of substrates and respiratory inhibitors in each mitochondrial preparation allowed the estimation of the fraction of electrons out of sequence which reduce oxygen to

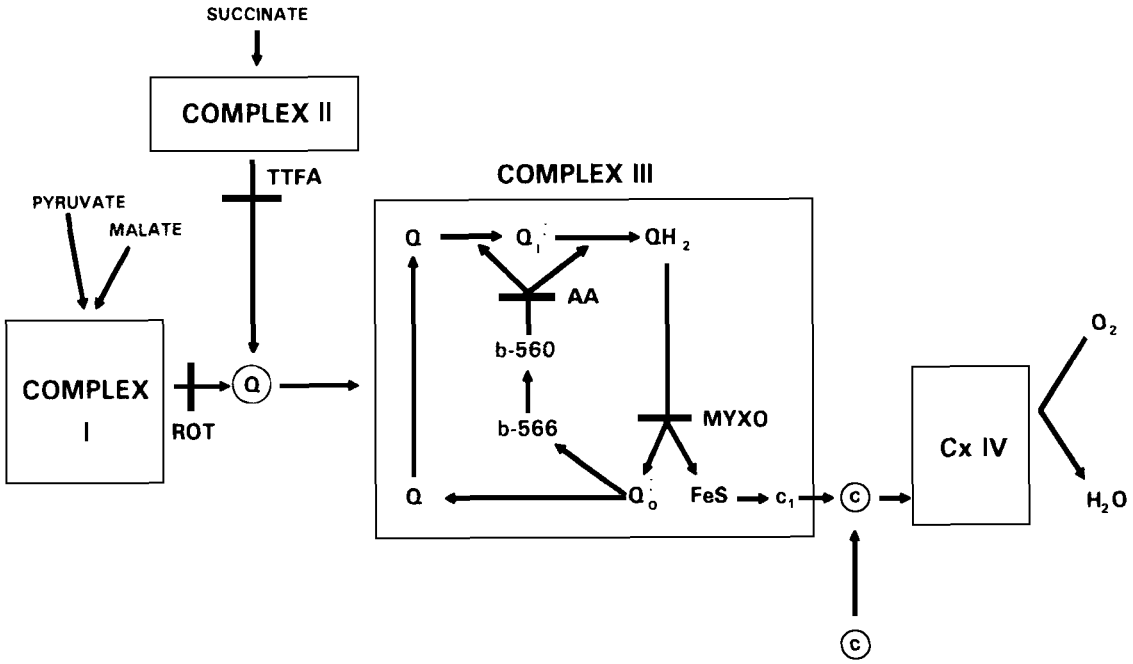

Fig. 1. The scheme shows the sites of action of specific substrates and inhibitors in relation to the four mitochondrial electron transport Complexes (I to IV). Pyruvate and malate are NADH-linked substrates which feed electrons at Complex I, whereas succinate introduces electrons at Complex II and cytochrome *c* does it between Complexes III and IV. Rotenone (ROT) inhibits electron flow from Complex I to the ubiquinone pool (Q). Thenoyltrifluoroacetone (TTFA) inhibits electron flow from Complex II to Q. Antimycin A (AA) blocks electron transport from cytochrome *b*-560 to Q or Q_i at center "in" (matrix side). Myxothiazol (MYXO) inhibits electron flow from ubiquinol (QH_2) to FeS center at Complex III. Q_i^- = center "in" semiquinone; Q_0 = center "out" (intermembrane side) semiquinone; FeS = iron sulfur center of Complex III; c_1 = cytochrome c_1; *c* = mobile cytochrome *c* pool; Cx IV = Complex IV.

oxygen radicals along the mitochondrial respiratory chain (the percent free radical leak) instead of reducing oxygen to water at the terminal cytochrome oxidase (Complex IV). Since two electrons are needed to reduce one molecule of oxygen to H_2O_2, whereas four electrons are needed to reduce one molecule of oxygen to water, the percent free radical leak was calculated as the rate of H_2O_2 production divided by two times the rate of oxygen consumption, and the result was multiplied by 100.

Statistical Analysis

The statistical significance of differences between rat and pigeon mitochondria, or between measurements in the absence and presence of a respiratory inhibitor, was studied with the Student's *t* test. The minimum statistical significance was set at $p < 0.05$.

RESULTS

Mitochondrial Free Radical Production

The rate of free radical production with pyruvate/malate as substrates alone was significantly higher in rat than in pigeon brain nonsynaptic mitochondria (Fig. 2). The maximum rates of free radical production observed in this work were obtained with Complex I (rotenone) or Complex III (myxothiazol and antimycin A) inhibitors in pyruvate/malate-supplemented mitochondria. Rotenone significantly increased free radical production with pyruvate/malate in both species (Figs. 2 and 3). The same was true for antimycin A and for myxothiazol, which increased the free radical production of rat and pigeon brain mitochondria to values not significantly different from those obtained with rotenone (Fig. 2). The significant difference between both animals in the rate of free radical production with pyruvate/malate alone—higher in the rat than in the pigeon—disappeared after addition of either rotenone,

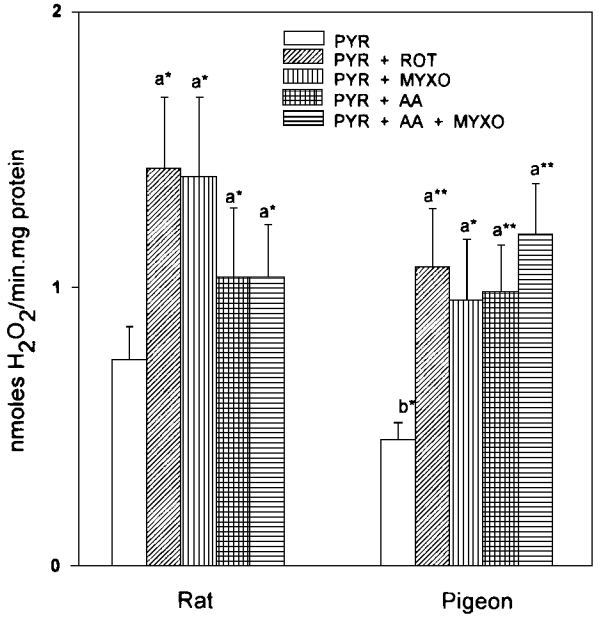

Fig. 2. Rate of free radical production of pyruvate/malate-supplemented rat and pigeon brain untreated mitochondria or treated with rotenone, myxothiazol, antimycin A, or antimycin A plus myxothiazol. In the experiments with inhibitors free radical production was first followed kinetically with substrate alone, then the inhibitor was added and the kinetics was continued in the same sample to observe its effect on the rate of H_2O_2 production. PYR = 5 mM pyruvate/2.5 mM malate; ROT = 2 μM rotenone; AA = 10 μM antimycin A; MYXO = 10 μM myxothiazol. Results are means ± SEM of 6 different animals. a* significantly different from pyruvate/malate alone in the same species; b* significantly different from rat values with pyruvate/malate alone; *$P < 0.05$; ** $P < 0.01$.

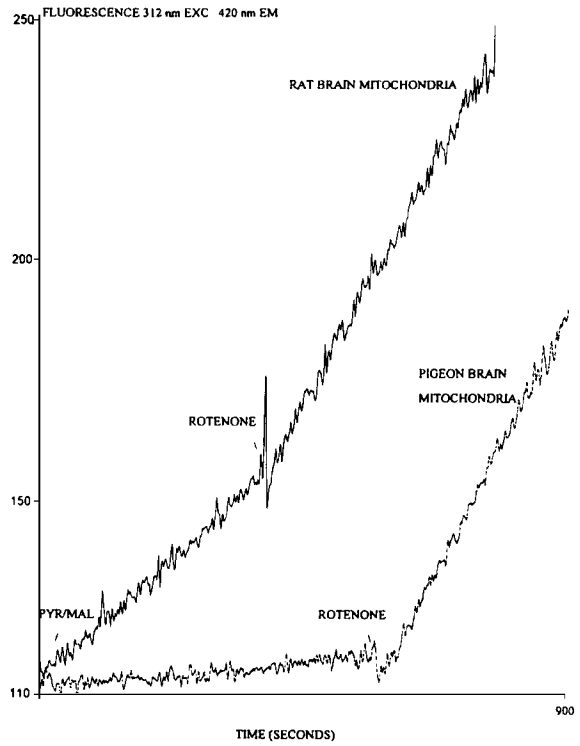

Fig. 3. Direct fluorometric tracings, showing the instantaneous increase in free radical production brought about by the addition of rotenone to rat and pigeon brain mitochondria supplemented with pyruvate/malate. The pigeon mitochondrial sample shown in the figure was the one showing the lowest rate among all the pigeon samples analyzed (see the lower difference in mean values between rats and pigeons in Fig. 2). Even in this particular pigeon sample showing an extremely low rate of free radical generation with substrate alone (not representative of the corresponding pigeon mean shown in Fig. 2), the addition of rotenone increased free radical production to a value similar to that observed in the rotenone-treated rat tracing (as it is statistically demonstrated for the mean values of Fig. 2). Free radical generation increases the fluorescence at 312 nm excitation and 420 nm emission due to H_2O_2 (see Materials and Methods for further details). Pyruvate 5 mM/malate 2.5 mM (PYR/MAL) were added and the kinetics were started (time 0). After some minutes rotenone was added and the kinetics were continued. The cuvette protein concentration was the same in both species (0.4 mg/ml), allowing direct comparison of both tracings. The transient perturbation on the tracings at the moment of rotenone addition are due to minimal opening of the sample compartment to add the inhibitor.

myxothiazol, or antimycin A (Fig. 2). Direct tracings of pyruvate/malate-supported free radical production (Fig. 3) show this for rotenone even in an individual pigeon brain mitochondrial sample showing extremely low values of free radical production (much lower than the mean for pigeon; see Fig. 2), since the rates became similar after rotenone addition. Myxothiazol addition to antimycin A-treated pyruvate/malate-supplemented brain mitochondria did not significantly change the rate of mitochondrial free radical production in either rats or pigeons (Fig. 2).

Succinate-supported free radical production of nonsynaptic brain mitochondria was one order of magnitude lower than that observed with pyruvate/malate in both rat and pigeon mitochondria supplemented with substrate alone ($p < 0.001$; Fig. 4). Neither thenoyltrifluoroacetone, antimycin A, or rotenone significantly changed free radical production in succinate-supported mitochondria from both species (Fig. 4).

Ferrocytochrome *c* was also used as substrate in brain mitochondria incubated during at least 10 min in hypotonic buffer in the presence of myxothiazol, a treatment which allows reduced cytochrome *c* to reach the respiratory chain at the inner membrane to direct electrons to Complex IV (Fig. 1). No free radical production was detected with ferrocytochrome *c* as substrate in either rat or pigeon mitochondria. The same

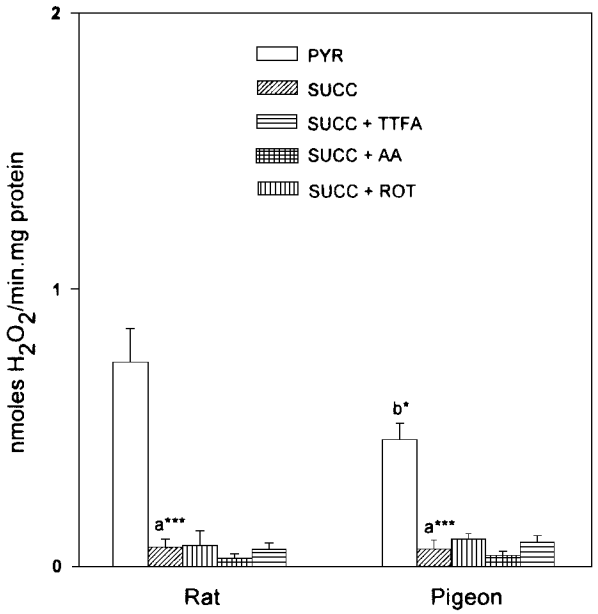

Fig. 4. Rate of free radical production of succinate-supplemented rat and pigeon brain untreated mitochondria or treated with thenoyltrifluoroacetone, antimycin A, or rotenone. In experiments with inhibitors the rate of free radical production was first followed kinetically with substrate alone, then the inhibitor was added and the kinetics was continued in the same sample to observe its effect. Rates with pyruvate/malate alone from Fig. 2 are included for comparison. PYR = 5 mM pyruvate/2.5 mM malate; SUCC = 10 mM succinate; TTFA = 11 μM thenoyltrifluoroacetone; AA = 10 μM antimycin A; ROT = 2 μM rotenone. Results are means ± SEM of five to six different animals. a* significantly different from pyruvate/malate alone in the same species; b* significantly different from rat values with pyruvate/malate alone; * $p < 0.05$; *** $p < P < 0.001$.

was true with ascorbate plus TMPD (tetramethylphenylene diamine) as substrates, which fed electrons between Complexes III and IV (results not shown).

Mitochondrial Oxygen Consumption

The rate of oxygen consumption was measured with pyruvate/malate, succinate, or ferrocytochrome *c* as substrates in brain mitochondria from both species (Table I). No significant differences in oxygen consumption between rat and pigeon mitochondria were observed in any case. Oxygen consumption was significantly higher with ferrocytochrome *c* than with pyruvate/malate or succinate in both rat and pigeon mitochondria (Table I). This result was expected in case of successful use of ferrocytochrome *c* as substrate, since pyruvate/malate feeds electrons before

Table I. Oxygen Consumption of Rat and Pigeon Brain Mitochondria Supplemented with Pyruvate/Malate, Succinate, or Ferrocytochrome *c*[a]

	Oxygen consumption (nmol O_2/min·mg protein)	
	Rat	Pigeon
Pyr/mal	6.5 ± 0.85	6.2 ± 1.6
Succinate	7.7 ± 1.9	7.5 ± 1.6
Ferrocytochrome *c*	21.3 ± 5.6*	14.2 ± 1.3**

[a] Pyr/mal (5 mM pyruvate/2.5 mM malate), succinate (10 mM), or ferrocytochrome *c* (24 μM) were used as substrates. Measurements with ferrocytochrome *c* were performed in hypotonic buffer (see Materials and Methods). No significant differences between rat and pigeon mitochondria were observed. Oxygen consumption values were significantly higher with ferrocytochrome *c* than those obtained with pyruvate/malate or with succinate in both rat (* $P < 0.05$) and pigeon mitochondria (** $P < 0.01$). Results are means ± SEM; number of different animals per mean = 6 (pyruvate/malate and succinate) or 4 (ferrocytochrome *c*).

energy coupling site 1 (at Complex I) and succinate does it before site 2, whereas ferrocytochrome *c* bypasses sites 1 and 2 when added in the presence of a Complex III inhibitor (myxothiazol in this work), thus only being able to feed electrons to energy coupling site 3 (at Complex IV). The nearer to the oxygen side is situated the entry of electrons to the respiratory chain, the higher must be the rate of oxygen consumption. Similarly to what happened for Complex I- and Complex II-linked substrates, the ferrocytochrome *c*-supplemented rates of oxygen consumption were not significantly different between rat and pigeon mitochondria.

Free Radical Leak in the Respiratory Chain

The free radical leak (the percentage of total mitochondrial electron flow directed to oxygen radical generation) was much higher with pyruvate/malate than with succinate and was significantly increased to maximum levels after addition of rotenone to pyruvate/malate-supplemented brain mitochondria in both animal species (Fig. 5). When supplemented with pyruvate/malate alone, the free radical leak was significantly higher in rat than in pigeon brain mitochondria (Fig. 5). Nevertheless, the difference in free radical leak between both species was no longer significant after rotenone addition to pyruvate/malate-supplemented mitochondria.

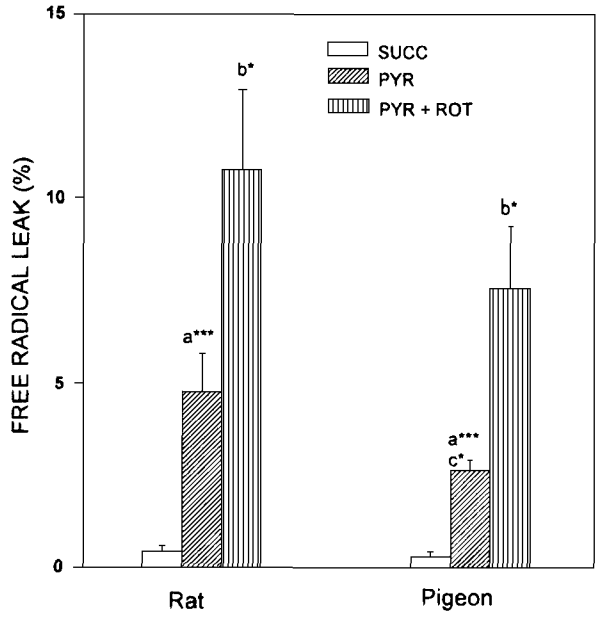

Fig. 5. Free radical leak in brain mitochondria from rats and pigeons supplemented with succinate, pyruvate/malate, or after addition of rotenone to pyruvate/malate-supplemented mitochondria. The free radical leak represents the fraction of the mitochondrial electron flow reducing oxygen to free radicals in relation to that reducing oxygen to water at cytochrome oxidase (see the corresponding Materials and Methods section for further details). SUCC = 10 mM succinate; PYR = 5 mM pyruvate/2.5 mM malate; ROT = 2 μM rotenone. Results are means ± SEM of six different animals. a* significantly different from succinate in the same species; b* significantly different from pyruvate/malate in the same species; c* significantly different from rat values with pyruvate/malate alone; * $p < 0.05$; *** $p < P < 0.001$.

DISCUSSION

In the present paper, all the respiratory chain inhibitors were used at concentrations which caused maximum effects on brain mitochondrial free radical production with minimum or no changes in the enzymatic H_2O_2 detection system. This ensures that their effects occur on the mitochondria themselves. Among factors affecting mitochondrial free radical production, the reduction state of autoxidizable electron carriers is of paramount importance (Chance, 1981). When the respiratory chain is blocked with an inhibitor, the reduction state of electron carriers on the substrate side is strongly increased, whereas those in the oxygen side become highly oxidized due to lack of electron flow. Therefore, an increase in mitochondrial free radical production following the addition of an inhibitor indicates that the site of free radical generation is situated on the substrate side in relation to the inhibitor. A decrease in free radical production following addition of the inhibitor means that free radical generator is located on the oxygen side.

Previous studies have detected (Cino and del Maestro, 1989; Barja *et al.*, 1994) or not (Sorgato *et al.*, 1974) free radical production with succinate in total brain mitochondria, and others have indirectly observed it in synaptic but not in nonsynaptic mitochondria (Floyd *et al.*, 1984). NADH-linked free radical production has been observed indirectly with malate/glutamate (Floyd *et al.*, 1984), or by ESR with Ca^{2+}-treatment (which increases matrix NADH) in brain mitochondria (Dykens, 1994), and with glutamate in forebrain neurons (Reynolds and Hastings, 1995). In the present investigation free radical production with NADH-linked substrate alone (pyruvate/malate) was directly demonstrated in rat and pigeon nonsynaptic brain mitochondria. The rate of free radical generation was much higher with pyruvate/malate than with succinate in both species. This suggests that the main free radical generation site of brain mitochondria is located in Complex I in both animal species, since the only difference in electron pathway between both kinds of substrates is the entry of electrons through Complex I (with pyruvate/malate) or through Complex II (with succinate).

Both antimycin A and myxothiazol, which inhibit electron flow within Complex III, strongly increased free radical production of pyruvate/malate-supplemented brain mitochondria in rats and pigeons. This indicates that the free radical generation site can be located at either Complex III or Complex I. Rotenone also increased free radical production with pyruvate/malate (and did not do it with succinate) in both species, demonstrating a main role of Complex I as free radical generator. The same has been found in bovine heart submitochondrial particles supplemented with NADH (Takeshige and Minakami, 1979; Turrens and Boveris, 1980). If Complex III were the main free radical generator, rotenone should have decreased, instead of increased, free radical production with pyruvate/malate. The rotenone-stimulated rates were not significantly different from those obtained with antimycin A or myxothiazol. This further indicates an absence of free radical production at Complex III. If both Complex I and Complex III were additively producing free radicals in pyruvate/malate-supplemented brain mitochondria treated with antimycin A or myxothiazol, a higher H_2O_2 generation would be expected with these inhibitors than with rotenone. Demonstration that Complex III is not involved in free

radical production in rat and pigeon brain nonsynaptic mitochondria also comes from the observations that: antimycin A increased free radical generation with pyruvate/malate, but did not do it with succinate (free radical production with succinate plus antimycin A has been observed in rat heart mitochondria, Boveris *et al*, 1976, but not in rat brain mitochondria, Sorgato *et al.*, 1974); myxothiazol did not decrease the antimycin A-stimulated rates of free radical production with pyruvate/malate in any species. This "double kill" experiment totally abolishes reduction of both b_{560} and b_{566} cytochromes and formation of center out ("cytosolic" side) semiquinone at Complex III (Matsuno-Yagi and Hatefi, 1996), since in this situation the electrons cannot reach these sites either trough center in (which is blocked by antimycin A) or center out (blocked by myxothiazol). Thus, the stimulation of free radical production in pyruvate/malate-supplemented nonsynaptic brain mitochondria by either antimycin or myxothiazol is also due to an increased reduction state of the free radical generation site at Complex I.

Complex II does not seem to contain an important free radical generator in nonsynaptic mitochondria since addition of TTFA to succinate-supplemented mitochondria did not increase H_2O_2 generation in either rats or pigeons. Incompletely reduced forms of oxygen are formed inside Complex IV as part of the cytochrome oxidase mechanism, but none of these free radical forms are considered to be released outside the Complex (Chance, 1981). However, direct demonstration of absence of free radical generation at Complex IV has been hampered by experimental limitations. We have not observed any free radical production in brain mitochondria incubated with a classical Complex IV substrate combination: ascorbate plus TMPD. But at the high ascorbate concentrations (millimolar range) needed in this assay ascorbate directly eliminates any H_2O_2 produced. Cyanide or azide cannot be used either, due to their inhibitory actions on the peroxidases included in the H_2O_2 detection systems. In our investigation, ferrocytochrome *c* was used as respiratory substrate for Complex IV in brain mitochondria incubated in hypotonic medium in the presence of myxothiazol, which was added to isolate the last segment of the respiratory chain. Ferrocytochrome *c* supported higher rates of oxygen consumption than succinate or pyruvate/malate (as expected if it was a successful substrate), without generating any detectable levels of free radicals. This indicates that Complex IV is not the main free radical generator in rat or pigeon brain mitochondria.

All the experiments performed in this work suggest that Complex I is the main free radical generator of rat and pigeon brain nonsynaptic mitochondria in state 4. This cannot be directly extrapolated to other tissues, since previous studies have shown that heart mitochondria respiring in state 4 (substrate alone) can generate free radicals both at Complex I (Takeshigue and Minakami, 1979; Turrens and Boveris, 1980; Herrero and Barja, 1997a) and at ubisemiquinone of Complex III (Boveris *et al.*, 1976; Cadenas and Boveris, 1980). Nevertheless, recent reports studying heart mitochondria mainly localized free radical production at Complex I in state 4 (Hansford *et al.*, 1997) and indicated that neither the concentration nor the main kind of ubiquinone present (Q-9 or Q-10) is responsible for the decrease in mitochondrial free radical production from short-lived to longevous mammalian species (Lass *et al.*, 1997). We have recently shown that Complex I is the only free radical generator in heart and nonsynaptic brain rat and pigeon mitochondria respiring in state 3 (substrate + ADP; Herrero and Barja, 1997b).

Maximum longevity is much higher in pigeons (35 years) than in rats (4 years) in spite of the similar specific basal metabolic rate and oxygen consumption of both species. Succinate-supported free radical production is lower in the pigeon than in the rat in crude mitochondrial fractions obtained from various organs (Barja *et al.*, 1994), and decreases as maximum longevity and body size increase in mammals from rat to cow (Sohal *et al.*, 1989, 1990; Ku *et al.*, 1993). The mechanisms which allow pigeon mitochondria to produce free radicals at low rate without decreasing oxygen consumption were previously unknown. The rate of free radical production can be decreased in the respiratory chain by at least two main mechanisms: (a) decreasing the rate of electron flow; or (b) decreasing the leak of free radicals per unit electron flow. The first mechanism can explain the low mitochondrial free radical production of longevous mammals of high body size (like the cow), since basal metabolic rate decreases as body size increases within an animal group, and the same is true for mitochondrial oxygen consumption in various mammals of different size from rat to cow (Ku *et al.*, 1993). But this mechanism does not take place in the case of pigeon brain mitochondria since they showed similar rates of oxygen consumption than rat brain mitochondria. The low free radical production of pigeon brain mitochondria with

substrate alone is due to a lower free radical leak in the respiratory chain, as demonstrated in this work. This would be possible if pigeon brain mitochondria are able to maintain a lower degree of reduction of the Complex I free radical generator than rat brain mitochondria. The effect of rotenone addition on pyruvate/malate-supplemented mitochondria demonstrates that this mechanism is operating. This treatment strongly increases the reduction state of Complex I and, in agreement with the mechanism proposed, abolishes the difference in free radical production and free radical leak between both species. Finally, it must be noted that the difference in free radical generation between rat and pigeon mitochondria was smaller than the difference in longevity between both species. This would be consistent with the widely held concept that aging rate is due to more than one single mechanism.

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REFERENCES

Ambrosio, G., Zweier, J. L., Duilio, C., Kuppusamy, P., Santoro, G., Elia, P. P., Tritto, I., Cirillo, P., Condorelli, M., Chiariello M., and Flaherty, J. T. (1993). *J. Biol. Chem.* **268**, 18532–18541.

Asuncion, J. G., Millan, A., Pla, R., Bruseghini, L., Esteras, A., Pallardo, F. V., Sastre, J., and Viña, J. (1996). *FASEB J.* **10**, 333–338.

Barja, G. (1998). In *Methods in Aging Research* (Yu, B. P., ed.), CRC Press, Boca Raton, Chapter 23, in press.

Barja, G., Cadenas, S., Rojas, C., Pérez-Campo, R., and López-Torres, M. (1994). *Free Radical Res.* **21**, 317–328.

Boveris, A., Cadenas, E., and Stoppani, O. M. (1976). *Biochem J.* **153**, 435–444.

Cadenas, E., and Boveris, A. (1980). *Biochem. J.* **188**, 31–37.

Calder, W. A. (1985). *Exp. Gerontol.* **20**, 161–170.

Chance, B. (1981). In *Oxygen and Living Processes* (Gilbert, D., ed.), Springer Verlag, New York, pp. 200–209.

Cino, M., and del Maestro, R. F. (1989). *Arch. Biochem. Biophys.* **269**, 623–638.

Dykens, J. A. (1994). *J. Neurochem.* **63**, 584–591.

Floyd, R. A., Zaleska, M. M., and Harmon, H. J. (1984). In *Free Radicals in Molecular Biology, Aging and Disease* (Aarmstrong, D., ed.), Raven Press, New York, pp. 143–161.

Hansford, R. G., Hogue, B. A., and Mildaziene, V. (1997) *J. Bioenerg. Biomembr.* **29**, 89–95.

Harman, D. (1994). *Age* **17**, 119–146.

Herrero, A., and Barja, G. (1997a). *Mech. Ageing Dev.* **98**, 95–11.

Herrero, A., and Barja, G. (1997b). *J. Bioenerg. Biomembr.* **29**, 243–251.

Holmes, D. J., and Austad, S. N. (1995). *Am. Zool.* **35**, 307–317.

Ku, H. H., Brunk, U. T., and Sohal, R. S. (1993). *Free Radical Biol. Med.* **15**, 621–627.

Lai C. K., and Clark, J. B. (1979). *Methods Enzymol.* **55**, 51–60.

Lass A., Agarwal, S., and Sohal, R. S. (1997). *J. Biol. Chem.* **272**, 19199–19204.

Matsuno-Yagi, A., and Hatefi, Y (1996). *J. Biol. Chem.* **271**, 6164–6171.

Nohl, H. (1994). *Ann. Biol. Clin.* **52**, 199–204.

Nohl, H., and Jordan, W. (1986). *Biochem. Biophys. Res. Commun.* **138**, 533–539.

Ozawa, T. (1995). *Exp. Gerontol.* **30**, 269–290.

Pearl R. (1928). In *The Rate of Living*, University of London Press, London.

Prothero, J., and Jürgens, K. D. (1987). In *Evolution of Longevity in Animals*, Plenum Press, New York, pp. 49–74.

Reynolds, I. J., and Hastings, T. G. (1995). *J. Neurosci.* **15**, 3318–3327.

Richter, Ch. (1995). *Curr. Topics Bioenerg.* **17**, 1–19.

Shapira, A. H. V. (1994). *Movement Disord.* **9**, 125–138.

Sherratt, H. S. A., Watmough, N. J., Johnson, M. A., and Turnbull, D. M. (1988). *Methods Biochem. Anal.* **33**, 304–305.

Shigenaga, M. K. H., and Ames, B. N. (1994). In *Natural Antioxidants in Health and Disease*, Academic Press, New York, pp. 63–106.

Sohal, R. S., Svensson, I., Sohal, B. H., and Brunk, U. T. (1989). *Mech. Ageing Dev.* **49**, 129–135.

Sohal, R. S., Svensson, I., and Brunk, U. T. (1990). *Mech. Ageing Dev.* **53**, 209–215.

Sorgato, M. C., Sartorelli, L., Loschen, G., and Azzi, A. (1974). *FEBS Lett.* **45**, 92–95.

Takeshige, K., and Minakami, S. (1979). *Biochem J.* **180**, 129–135.

Turrens, J. F., and Boveris, A. (1980). *Biochem. J.* **191**, 421–427.

Yu, B. P., (1996). *Free Radical Biol. Med.* **21**, 651, (1996).