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ADP-Regulation of Mitochondrial Free Radical Production Is Different with Complex I- or Complex II-Linked Substrates: Implications for the Exercise Paradox and Brain Hypermetabolism

A. Herrero[1] and G. Barja[1,2]

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In agreement with classic studies, succinate-supplemented rat and pigeon heart and nonsynaptic brain mitochondrial free radical production is stopped by ADP additions causing the stimulation of respiration from State 4 to State 3. Nevertheless, with Complex I-linked substrates, mitochondria produce free radicals in State 3 at rates similar or somewhat higher than during resting respiration. The absence of sharp increases in free radical production during intense respiration is possible due to strong decreases of free radical leak in State 3. The results indicate that Complex I is the main mitochondrial free radical generator in State 3, adding to its already known important generation of active oxygen species in State 4. The observed rate of mitochondrial free radical production with Complex I-linked substrates in the active State 3 can help to explain two paradoxes: (a) the lack of massive muscle oxidative damage and shortening of life span due to exercise, in spite of up to 23-fold increases of oxygen consumption together with the very low levels of antioxidants present in heart, skeletal muscle, and brain; (b) the presence of some degree of oxidative stress during exercise and hyperactivity in spite of the stop of mitochondrial free radical production by ADP with succinate as substrate.

KEY WORDS: ADP; mitochondria; free radical production; brain; heart; exercise; hypermetabolism.

INTRODUCTION

Mitochondrial free radical production is probably involved in physiological processes like aging, exercise, and variations of cellular metabolic rate (Harman, 1994; López-Torres *et al.*, 1993; Barja *et al.*, 1990, 1991, 1994). In healthy tissues the majority of reactive oxygen species are produced at mitochondria, which are responsible for more than 90% of oxygen consumption. Nevertheless, few studies about the physiological factors which regulate the rate of mitochondrial free radical production have been performed.

It is currently considered that the two major parameters controlling respiration in cells are the reduced equivalent supply and the cytosolic adenine nucleotides (Brand and Murphy, 1987; Balaban, 1990). High or small increases in cytosolic ADP concentration stimulate mitochondrial oxidative phosphorylation depending on the tissue or situation including moderate or strenuous exercise or hypermetabolism (Newby *et al.*, 1990; Bangsbo *et al.*, 1993; Kushmerick, 1994; Dobson and Headrick, 1995). Early studies found that succinate-supported free radical production of heart mitochondria is stopped after the addition of sufficient ADP to cause the energy transition from the resting State 4 to the respiratorily active State 3 (Loschen *et al.*, 1971; Boveris *et al.*, 1972). This finding generated the widespread notion that mitochondria only produce free radicals in the resting State 4 condition. Mitochon-

[1] Department of Animal Biology-II (Animal Physiology), Faculty of Biology, Complutense University, Madrid 28040, Spain.
[2] To whom correspondence should be addressed.

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dria in cells are often described as being in a state intermediate between States 4 and 3 (Brand and Murphy, 1987), and in continuously working aerobic tissues like the heart the mitochondrial state is considered to be closer to State 3 than to State 4. A lack of free radical generation in State 3 would eliminate the implication of mitochondrial reactive oxygen species in tissue damage during exercise and hypermetabolism. But increases in free radical concentrations have been detected by direct ESR techniques in rat and human muscle during exercise (Davies *et al.*, 1982; Jackson *et al.*, 1985; Barja, 1992) and in neurons during hypermetabolic excitotoxicity (Dugan *et al.*, 1995; Reynolds and Hastings, 1995). This constitutes a first "exercise" paradox.

The depleting effect of ADP on mitochondrial free radical production was typically observed with a Complex-II linked substrate (succinate; Loschen *et al.*, 1971, 1973; Boveris *et al.*, 1972; Cadenas and Boveris, 1980) which by-passes Complex I—a main mitochondrial free radical generator (Takeshige and Minakami, 1979; Turrens and Boveris, 1980)—whereas the majority of the reducing equivalents enter the brain and heart respiratory chain through Complex I. In this work the control by ADP of free radical production and free radical leak were studied using both Complex II (succinate)- and Complex I (pyruvate/malate)-linked substrates in heart and nonsynaptic brain mitochondria. Since this parameter seems to be related to the longevity of the donor species (Ku *et al.*, 1993; Barja *et al.*, 1994; Sohal and Weindruch, 1996), homeothermic animals with very different maximum longevities—4 years in rats and 35 years in pigeons—were used.

The simple idea that the rate of mitochondrial free radical production increases in proportion to the rate of oxygen consumption is frequently assumed without evidence about it. Tissue oxygen consumption increases up to 23-fold in human skeletal muscle and 3.6-fold in the human heart from rest to heavy work, heart maximum VO_2 values (18 ml of O_2/g·h) being the greatest of the human body and fivefold higher than those of skeletal muscle in maximum aerobic exercise (Tyler, 1992). But cardiac and skeletal muscles have antioxidant concentrations more than one order of magnitude lower than those of other highly aerobic tissues like liver or kidney, a trait also shared by the enzymatic H_2O_2 detoxifying capacity of the brain. It is then difficult to understand how muscles could avoid a massive oxidative damage during an acute exercise bout—a situation which does not take place—if free radical production would immediately increase by 23-fold during exercise. This constitutes a second "excercise" paradox. The results obtained here, showing that a moderate free radical production occurs with Complex I-linked substrates during ADP-stimulated mitochondrial active respiration (State 3), can help to explain the two exercise or hypermetabolic paradoxes.

MATERIALS AND METHODS

Isolation of Heart and Nonsynaptic Brain Mitochondria

Male Wistar rats 6–8 months of age were obtained from Iffa-Creddo (Lyon, France) and male pigeons (*Columba livia*) 2–4 years of age were obtained from a Spanish breeder. Both animal species were acclimated in the laboratory during at least one week at 25°C and 12:12 L:D illumination conditions. Animals were sacrificed by decapitation.

Ventricles were quickly excised and chilled, fat was eliminated, and the tissue was rinsed, chopped, and homogenized in a Dounce with loose fitting pestle in 10 ml of isolation buffer (220 mM mannitol, 70 mM sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4) containing 5 mg of nagarse and 25 mg of fatty acid-free albumin. After standing for 1 min, 25 ml of additional isolation buffer containing 25 mg of albumin were added and homogenization was performed again with a tighter-fitting pestle. After homogenization, the pH was checked and readjusted to 7.4 if needed. Mitochondria were isolated by serial centrifugations at 700 *g* during 10 min (obtaining supernatants) and at 8,000 *g* during 10 min (obtaining pellets). The final mitochondrial pellets were resuspended in 1 ml of isolation buffer.

Nonsynaptic brain mitochondria were isolated by centrifugation in discontinuous 3%/6% Ficoll gradients following the method of Lai and Clark (1979) with the following modifications: (a) the 6% Ficoll medium was carefully underlayered below the 3% Ficoll; (b) an additional last resuspension in 35 ml of isolation medium (250 mM sucrose, 0.5 mM K^+-EDTA, 10 mM Tris-HCl, pH 7.4) followed by centrifugation at 11,500 *g* for 10 min was performed to eliminate the Ficoll from the final mitochondrial suspension.

All the procedures described above for isolation of heart or brain mitochondria were performed at 5°C in a RC5C Sorvall centrifuge with a SS-34 rotor or in the cold room. Mitochondrial protein was measured

by the Biuret method. The final mitochondrial suspensions were maintained over ice and were used for the oxygen consumption and free radical production measurements no later than 2h afterwards.

Mitochondrial Oxygen Consumption

The rate of oxygen consumption of heart and brain mitochondria was measured at 30°C in a water-thermostted incubation chamber with a Clark-type O_2 electrode and O_2 electrode and O_2 control box (CB1-D Hansatech) in 0.5 ml of incubation medium (145 mM KCl, 30 mM Hepes, 5 mM KH_2PO_4, 3 mM $MgCl_2$, 0.1 mM EGTA, pH 7.4) with 0.25 mg of mitochondrial protein per ml and succinate (5 mM—heart or 10 mM—brain) or pyruvate/malate (2.5 mM each—heart or 5 mM/2.5 mM—brain) as substrates, in the absence (State 4) followed by in the presence (State 3) of 500 μM ADP. The incubation medium was constantly stirred with a built-in electromagnetic stirrer and bar flea. After correction for nonmitochondrial oxygen consumption (with 10 μM myxothiazol for pyruvate/malate and with 10 μM antimycin A for succinate) respiratory control ratios higher than 5.0 (pyruvate/malate) and between 2.3 and 3.4 (succinate) were obtained in both species.

Mitochondrial Oxygen Radical Production

The rate of mitochondrial free radical production was measured following the linear increase in fluorescence (excitation at 312 nm, emission at 420 nm) due to enzymatic oxidation of homovanilic acid by H_2O_2 in the presence of horseradish peroxidase (Ruch *et al.*, 1983), modified to kinetically follow the rate of production of reactive oxygen species by isolated mitochondria (Barja *et al.*, 1994) in a LS50B computer-controlled Perkin-Elmer fluorometer. Reaction conditions were 0.25 mg (heart) or 0.4 mg (brain) of mitochondrial protein per ml, 6U/ml of horseradish peroxidase, 0.1 mM homovanilic acid, 50 U/ml of superoxide dismutase (SOD), and succinate or pyruvate/malate (concentrations as in oxygen consumption assays) added at the end to start the reaction

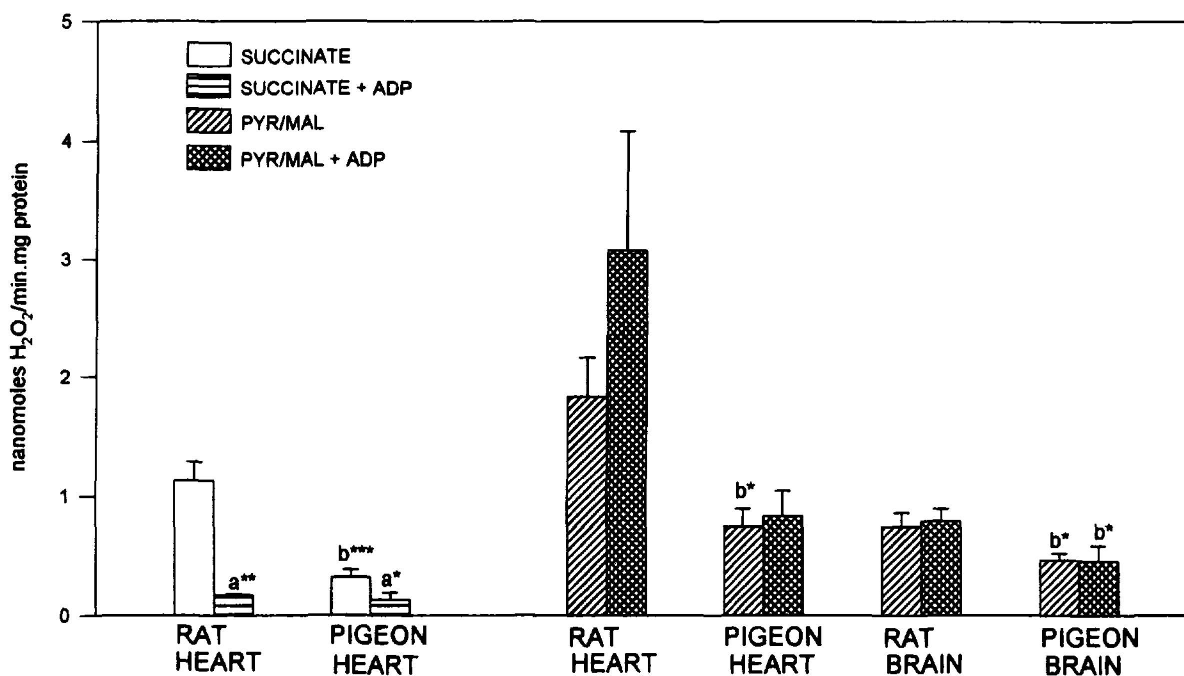

Fig. 1. Rates of State 4 (substrate alone) and State 3 (substrate plus ADP) free radical production of rat and pigeon heart and nonsynaptic brain mitochondria supplemented with Complex I- or Complex II-linked substrates. Production of H_2O_2 was first followed kinetically with substrate alone. After some minutes ADP was added to a 500 μM final concentration and the kinetics was continued in the same sample to observe the ADP effect. PYR/MAL = pyruvate/malate; ADP (500 μM). Values are means ± SEM from 6 to 7 different animals. Asterisks represent significant differences: a = ADP effect; b = difference between rats and pigeons under the same conditions; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

in the same incubation buffer used for the measurements of mitochondrial oxygen consumption at 30°C. All the assays were performed in the presence of SOD and thus the measured rates estimate the total (O_2^- plus H_2O_2) mitochondrial production of oxygen radicals. 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 of protein. The fluorometer cuvette holder was water-thermostted and the temperature of the sample was continuously monitored with a built-in sensor in the cuvette holder plus computer correction for holder—sample thermal difference. The cuvette medium was constantly stirred with a built-in electromagnetic stirrer (biokinetic accessory of the LS50B) and a bar flea. This allowed the rapid mixing of ADP, which was added at 500 μM final concentration during each kinetic experiment by slightly opening the sample compartment. An event marker indicated the exact time of addition of ADP in each experiment. The slope of the linear increase in fluorescence with time obtained after addition of substrates or ADP was measured performing a software-assisted least-squares linear regression which takes into account all the relevant data points between selected time windows. This allowed the elimination of initial time lags after substrate addition or final flat saturated tracings (which can occasionally occur in very active samples), if present.

Mitochondrial Free Radical Leak

Free radical production and oxygen consumption of heart and brain mitochondria were measured in the same buffer, using the same concentrations of substrates and ADP, and were assayed at the same temperature. This allowed the calculation of the fraction of electrons out of sequence which reduce O_2 to oxygen free radicals at the respiratory chain (the percent free radical leak) instead of reaching Complex IV to reduce O_2 to water. Since two electrons are needed to reduce one mole of O_2 to H_2O_2 whereas four electrons are transferred in the reduction of one mole of O_2 to water, the percent free radical leak was calculated as the rate of free radical production divided by two times the rate of O_2 consumption, and the result was multiplied by 100.

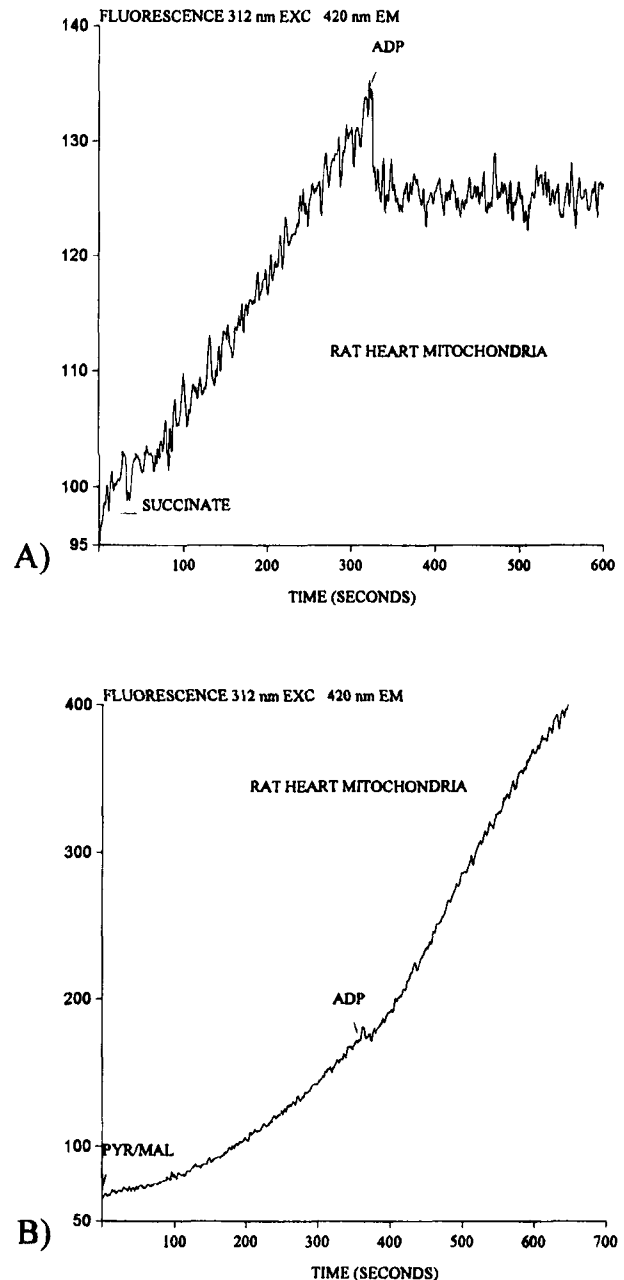

Fig. 2. Direct tracings showing the effect of ADP on free radical production of rat heart mitochondria with Complex II- (A) or Complex I-linked (B) substrates during the stimulation of oxygen consumption from State 4 to State 3. Accumulation of H_2O_2 increases the fluorescence at 312 nm excitation and 420 nm emission. PYR/MAL = pyruvate/malate; ADP (500 μM). Substrate was added and the kinetics was started (time 0). After some minutes, addition of ADP stopped free radical production with succinate (A) but not with pyruvate/malate (B). Transient perturbation of the tracings at the moment of ADP addition are due to slightly opening the sample compartment to add ADP. The tracing shown in (B) corresponds to one of the mitochondrial samples which responded to ADP with an increase in the rate of free radical production (see text).

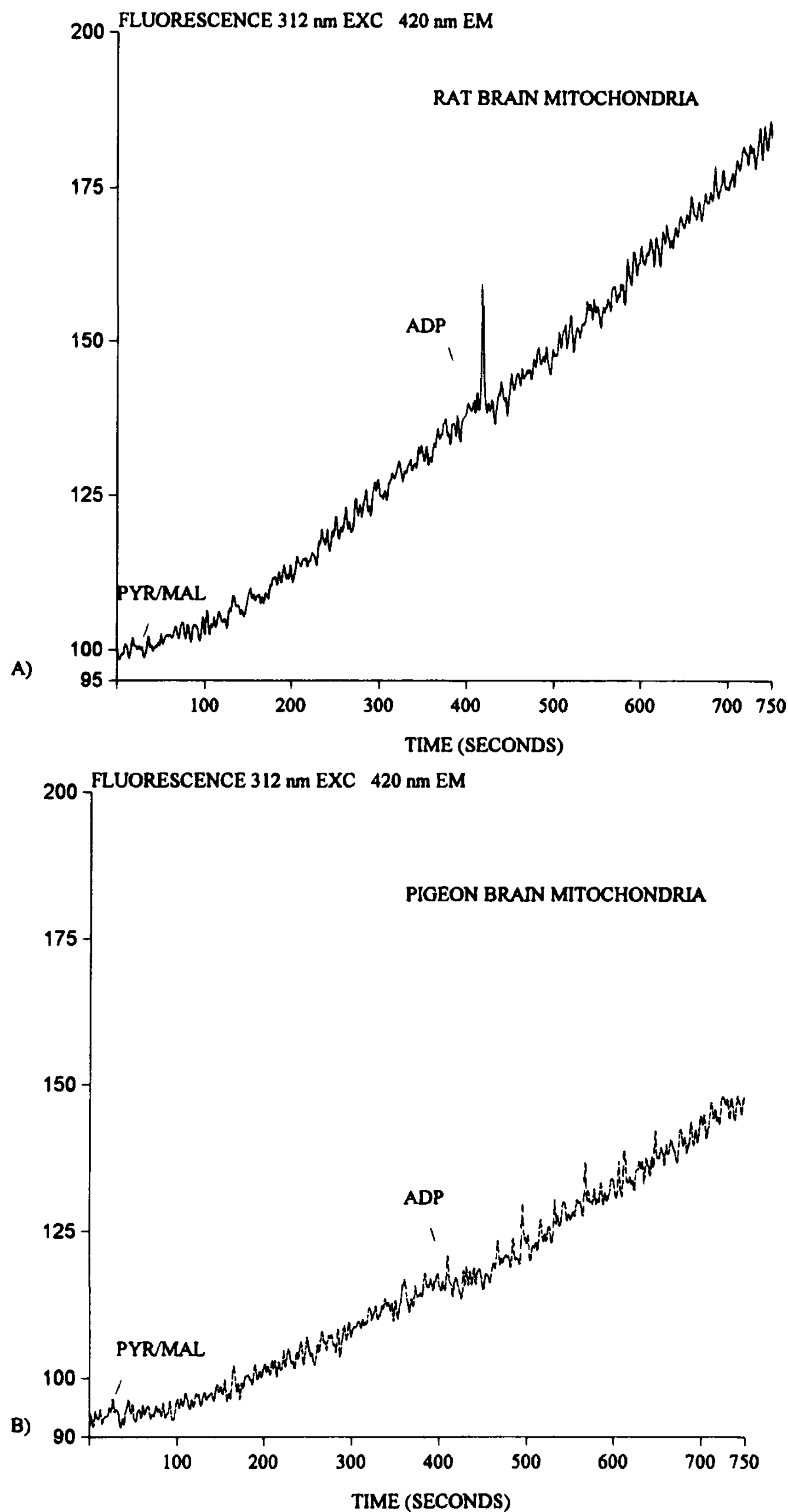

Fig. 3. Direct tracings showing the effect of ADP on free radical production of rat (A) and pigeon (B) nonsynaptic brain mitochondria with Complex I-linked substrates during the stimulation of oxygen consumption from State 4 to State 3. Accumulation of H_2O_2 increases the fluorescence at 312 nm excitation and 420 nm emission. PYR/MAL = pyruvate/malate; ADP (500 μM). Substrate was added and the kinetic was started (time 0). After some minutes, addition of ADP did not change the rate of free radical production in either species. Transient perturbations of the tracings at the moment of ADP addition are due to slightly opening the sample compartment to add ADP.

Statistical Analysis

Differences in mitochondrial free radical production, oxygen consumption, or free radical leak between means in the absence or presence of 500 μM ADP were statistically analyzed with Student's *t* tests. The same was done for differences between animal species under the same assay conditions. The minimum level of statistical significance was set at $p < 0.05$ in all the analyses.

RESULTS

The rate of free radical production of heart mitochondria with succinate alone (State 4) was significantly higher in the rat than in the pigeon (Fig. 1). Addition of ADP increased oxygen consumption by releasing the phosphorylating State 3 respiration. This ADP addition resulted in a strong decrease in the rate of succinate-supported free radical production of heart mitochondria in State 3 in both animal species (Fig. 1). The ADP-inhibiting effect was more acute in rat heart mitochondria—depletion to 15% of the values observed in State 4—than in the already low free radical production of pigeon heart mitochondria which decreased to 40% of State 4 values. As a result, in State 3 no differences in free radical production were observed between both species (Fig. 1). A direct tracing showing the virtual stop of rat heart mitochondrial free radical production induced by the ADP addition during the State 4 to State 3 transition is shown in Fig. 2A.

Contrary to what happened with succinate as substrate, the rate of free radical production of pyruvate/malate-supplemented heart mitochondria was not stopped by addition of ADP and continued unabated in State 3 both in rats and pigeons (Fig. 1), even though ADP increased the total oxygen consumption of the mitochondrial preparations by a factor of 3 to 4. In rat heart mitochondria the mean values of free radical production with pyruvate/malate were even higher, although not significantly, in the presence than in the absence of ADP (Fig. 1). This lack of statistical significance was due to the high variability observed in this case in the response to ADP among samples from different animals, since half of them reacted with 60 to 129% increases and half with small (10–20%) or no increases in free radical production. Figure 2B shows a direct tracing from the sample showing stronger increases in pyruvate/malate-supplemented free radical production after ADP addition. Decreases in free radical production were not observed in any heart sample studied with pyruvate/malate. In State 4, pyruvate/malate-supported free radical production of heart mitochondria was significantly higher in rats than in pigeons (Fig. 1).

Nonsynaptic brain mitochondria supplemented with succinate produced very low levels of free radicals which were near to the limit of detection or did not generate detectable free radicals (results not shown). This avoided the study of the ADP-depleting effect observed in succinate-supplemented heart mitochondria. Nonsynaptic brain mitochondria showed rates of free radical production with pyruvate/malate in States 3 and 4, which were significantly higher in rats than in pigeons (Fig. 1). Similarly to what happened in heart mitochondria, addition of ADP (State 4 to 3 transition) to pyruvate/malate-supplemented nonsynaptic brain mitochondria did not significantly change the rates of free radical production in either rats or pigeons (Fig. 1 and 3).

The fraction of electrons out of sequence which reduce O_2 to active oxygen species at the respiratory chain—the percent free radical leak—was calculated by relating the rates of free radical production to those of oxygen consumption (see Materials and Methods) under either State 4 or State 3 conditions. State 4 oxygen consumption and free radical production of heart mitochondria were significantly lower in pigeons than in rats with both kinds of substrates. Nevertheless, the free radical leak of heart mitochondria in State 4 was still lower in the pigeon than in the rat in both cases (Fig. 4). Addition of ADP strongly decreased the free radical leak of heart mitochondria with both kinds of substrates in rats and pigeons: from 2–4% to around 1.4% with pyruvate/malate and from 0.6–1.3% to very low values (around 0.1% of total electron flow) with succinate (Fig. 4). This was due to the decrease (succinate) or moderate or no increase (pyruvate/malate) of free radical production together with the strong stimulation of oxygen consumption during the State 4 to State 3 transition. Addition of ADP eliminated the differences in free radical leak of heart mitochondria (higher in the rat with both substrates) between both animal species, which were reduced to minimum values around 0.1% (succinate) or 1% (pyruvate/malate) of total electron flow (Fig. 4). The decrease in free radical leak during the State 4 to State 3 transition was higher in rat (70%) than in pigeon (40%) heart mitochondria with pyruvate/malate, and the same was true with succinate as substrate (95% decrease in the rat and 79% decrease in the pigeon).

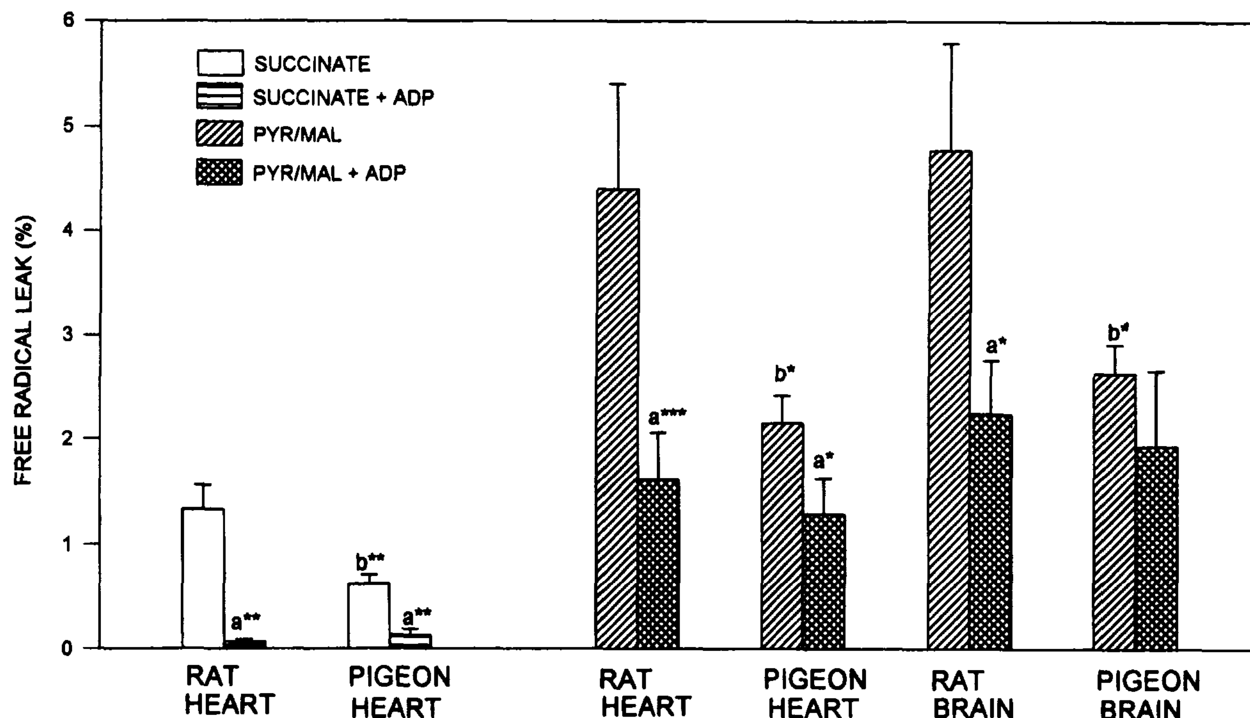

Fig. 4. Free radical leak (%) of State 4 (substrate alone) and State 3 (substrate plus ADP) rat and pigeon heart and nonsynaptic brain mitochondria supplemented with Complex I- or Complex II-linked substrates. The free radical is the percentage of total electron flow reducing oxygen to oxygen radicals at the respiratory chain. PYR/MAL = pyruvate/malate; ADP (500 μM). Values are means ± SEM from 6 to 7 different animals. Asterisks represent significant differences: a = ADP effect; b = difference between rats and pigeons under the same conditions; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Oxygen consumption values (States 4 and 3) were similar in rats and pigeons in pyruvate/malate-supplemented nonsynaptic brain mitochondria (results not shown). Thus, the significantly lower free radical leak of pigeon brain mitochondria (Fig. 4) was the only cause of the lower free radical production observed in the bird in State 4 (Fig. 1). Addition of ADP significantly decreased the free radical leak in rat brain mitochondria (Fig. 4). This was due to the increase in oxygen consumption during the State 4 to State 3 transition since free radical production was not changed by ADP (Fig. 1). The values obtained in pigeon brain mitochondria were suggestive of a similar (although smaller) ADP effect even though the ADP-induced trend to a decrease in free radical leak did not reach statistical significance in this case (Fig. 4). Similarly to what happened in heart mitochondria, addition of ADP eliminated the differences in free radical leak between both animal species.

DISCUSSION

The majority of (although not all) previous works on the regulation of mitochondrial free radical production by ADP or uncouplers have used succinate as substrate, rather than pyruvate, amino acids, ketone bodies, or fatty acids, the main physiological sources of reducing equivalents in most cells including cardiomiocytes and neurons. These last substrates finally feed electrons to mitochondria mostly via Complex I.

In this work, the classic description of the stop by ADP of free radical production in succinate-supplemented mitochondria (Loschen *et al.*, 1971; Boveris *et al.*, 1972) was observed in heart mitochondria from rats and pigeons. Nevertheless, we found that ADP does not stop free radical production with Complex-I linked substrates (pyruvate/malate) in either heart or nonsynaptic brain mitochondria from both animal species. This is consistent with previous observations in mitochondria respiring with malate/glutamate (Boveris *et al.* 1972) or succinate/glutamate (Boveris and Chance, 1973), with recent detection by ESR of free radical production in glutamate/malate-supplemented cerebral mitochondria exposed to Na^{+} and Ca^{2+} in the presence of ADP (Dykans, 1994), and with production of reactive oxygen species in glutamate- or *N*-methyl-D-aspartate-stimulated intact neurons (Dugan *et al.*, 1995; Reynolds and Hastings, 1995). In our case, ADP

even stimulated free radical generation with pyruvate/malate in various rat heart mitochondria samples. In agreement with the free radical theory of aging, State 4 rates of free radical production were lower in pigeon than in rat mitochondria. Nevertheless, the effects of ADP on mitochondrial free radical production were similar in rats and pigeons irrespective of their very different longevities.

Pyruvate/malate respiration of heart mitochondrial preparations was stimulated by 3- to 4-fold in our work during the State 4 to 3 transition. This is similar to the magnitude of the respiratory stimulation of the heart from rest to heavy work *in vivo* (Tyler, 1992). Measurement of mitochondrial free radical production has routinely been performed in the absence of ADP in many previous works, whereas ADP is present in resting tissues *in vivo* at low μM concentrations as calculated from ^{31}P-NMR data (Kemp *et al.*, 1993). This stresses the relevance of the observation of free radical production even in the presence of saturating ADP levels (500 μM) in pyruvate/malate supported mitochondria.

The presence of ADP strongly decreased the percentage of total electron flow escaping from the respiratory chain to reduce O_2 to oxygen radicals—the mitochondrial free radical leak—in almost every tissue and species. In the case of succinate this was mainly due to the depleting effect of ADP on free radical production. With Complex-I-linked substrates, the lower free radical leak in State 3 than in State 4 was due to continuation or to a relatively moderate stimulation of free radical production while oxygen consumption and total electron flow were more intensely stimulated. The decreased free radical leak in State 3 can be a protecting factor against acute oxidative stress during intense increases in respiration of aerobic tissues. The global effects of ADP on free radical production with both kinds of substrates can possibly explain in part the two apparent exercise (or hypermetabolic) paradoxes outlined in the justification of this work: (a) there can be some oxidative damage during acute exercise—especially if untrained—and tissue activation because State 3 mitochondria produce free radicals with Complex I-linked substrates in this State; (b) oxidative damage is not massive during activity, in spite of the strong increment in mitochondrial oxygen consumption and the low antioxidant capacity of muscles (Barja, 1992; Jenkins *et al.*, 1985), heart (Rojas *et al.*, 1994, 1996), and brain (Barja *et al.*, 1990), because the mitochondrial free radical leak strongly decreases during the State 4 to State 3 transition. The lack of massive increases in mitochondrial free radical production in State 3 would be consistent with the observation that chronic exercise does not decrease longevity neither in rodents (Goodrick, 1980; Holloszy *et al.*, 1985) or in humans (Paffenbarger *et al.*, 1986; Lee *et al.* 1995).

It is well established that the rate of mitochondrial free radical production rises strongly when the degree of reduction of the respiratory chain electron carriers is increased (Loschen *et al.*, 1971; Boveris *et al.*, 1972). Treatment of succinate-supplemented rat or pigeon heart mitochondria, and rat brain or liver mitochondria with many different uncouplers such as pentachlorophenol (Loschen *et al.*, 1971; Boveris *et al.*, 1972), FCCP (Boveris and Chance, 1973), or CCCP (Cino and Del Maestro, 1989), similarly to what happened with ADP, stopped free radical production. On the other hand, using different substrates and inhibitors of the respiratory chain, it has been found that Complex I and Complex III are the sites of mitochondrial free radical generation in State 4 (Hinkle *et al.*, 1967; Takeshige and Minakami, 1979; Cadenas and Boveris, 1980; Turrens and Boveris, 1980; Turrens *et al.*, 1985; Nohl and Jordan, 1986; Cino and Del Maestro, 1989).

The degree of reduction of the respiratory chain strongly decreases during the State 4 to State 3 transition at the same time that the rate of electron flow is increased (Chance and Williams, 1956; Tzagoloff, 1982). Our results show that a pyruvate /malate-supplemented heart and nonsynaptic brain rat and pigeon mitochondria avoid massive increases in free radical production during State 3 active respiration owing to their strong decrease in free radical leak. This probably results to a large extent from a lower degree of reduction of the Complex I free radical generator during intense electron flow in State 3. Free radical production with Complex I-linked substrates is maintained at comparable rates in States 3 and 4 since the decrease in free radical leak compensates for the increase in electron flow in State 3. The close proximity to the substrate probably allows a degree of reduction of the Complex I free radical generator still high enough for continuation of free radical production in State 3 whereas the Complex III generator, which is situated further away from the substrate, stops producing free radicals in the active state. Since ADP stops free radical production with succinate but not with pyruvate/malate, Complex I must be the main or only source of oxygen radicals in State 3 because Complexes III and IV are implicated in electron flow with both kinds of substrates and Complex II participates only when

free radical production stops (with succinate). Thus, Complex I is a main free radical generator both in the resting (State 4) and in the active (State 3) mitochondrial state. The stronger decrease in free radical leak in rat than in pigeon heart and brain pyruvate/malate-supplemented mitochondria during the State 4 to State 3 transition to similarly low values in both species also suggests that the relatively higher mitochondrial free radical production of the short-lived species (the rat) in State 4 is due, at least in part, to a higher degree of reduction of Complex I in the rodent than in the bird.

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