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Minireview

The Quantitative Measurement of H_2O_2 Generation in Isolated Mitochondria

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Adequate methods to measure the rate of mitochondrial oxygen radical generation are needed since oxygen radicals are involved in many pathologies. A fluorometric method appropriate to measure the rate of generation of H_2O_2 in intact mitochondria is described. Just after isolation of functional mitochondria from fresh tissues, rates of generation of H_2O_2 are kinetically measured by fluorometry in the presence of homovanillic acid and horseradish peroxidase. The method is specific for H_2O_2 and is sensitive enough to assay mitochondrial H_2O_2 generation in the presence of respiratory substrate without inhibitors of the respiratory chain. Simultaneous measurement of mitochondrial oxygen consumption allows calculation of the free radical leak: the percentage of electrons out of sequence which reduce oxygen to oxygen radicals along the mitochondrial respiratory chain instead of reducing oxygen to water at the terminal cytochrome oxidase. The method shows instantaneous response to H_2O_2. This makes it appropriate to study the quick effects of different inhibitors and modulators on the rate of mitochondrial oxygen radical production. Its application to the localization of the sites where caloric restriction decreases mitochondrial oxygen radical generation in heart mitochondria is described.

KEY WORDS: H_2O_2; hydrogen peroxide; mitochondria; free radicals; heart; superoxide; oxygen radicals.

INTRODUCTION

Aerobic tissues continuously generate reactive oxygen species (ROS) which can damage all kinds of macromolecules. This can be related to the development of many different kinds of degenerative diseases. The most widespread main oxygen radical generator of aerobic tissues is the mitochondrial respiratory chain, responsible for more than 90% of cellular oxygen consumption. Thus, accurate assays of mitochondrial oxygen radical generation are needed. It is generally considered that the respiratory chain univalently reduces oxygen to $O_2^{\cdot-}$. This $O_2^{\cdot-}$ dismutates to H_2O_2 which is then secreted by mitochondria to their surrounding medium. In submitochondrial particles, however, $O_2^{\cdot-}$ is secreted. Thus, the rate of oxygen radical generation by the respiratory chain is assayed as $O_2^{\cdot-}$ production in submitochondrial particles and as H_2O_2 production in intact mitochondria. Submitochondrial particles are convenient for various reasons such as the direct use of NADH as substrate or to keep the mitochondrial samples frozen for later measurements, whereas assays in intact functional mitochondria are closer to a physiological situation.

ROS DETECTION ASSAYS

Various methods using chemiluminescence, electron spin resonance (ESR), spectrophotometry, or fluorometry, have been used to estimate ROS levels in cells, mitochondria, or submitochondrial particles. Methods with high sensitivity and specificity are needed because the amount of ROS produced by intact mitochondria respiring with substrate alone is small. Chemiluminescent methods are among those showing higher sensitivities, but they lack chemical specificity, usually require long integration time intervals, and are then scarcely used for this purpose (Bates *et al.*, 1994; Chance and Gao, 1994). Direct or spin-trap

Key to abbreviations: H_2O_2, hydrogen peroxide; ROS, reactive oxygen species; SOD, superoxide dismutase; ESR, electron spin resonance; DCF, 2′,7′-dichlorofluorescin.

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ESR techniques can have enough specificity and have been occasionally used to assay ROS levels in brain mitochondria (Dykens, 1994) and other frozen (Ambrosio *et al.*, 1993) or unfrozen cellular fractions (Dugan *et al.*, 1995; Giulivi *et al.*, 1995). However, the sensitivity of these techniques is limited, and they require expensive equipment. It is useful to have appropriate methods to measure ROS production using instruments commonly present in biochemistry laboratories.

The measurement of $O_2^{\cdot -}$ production by spectrophotometry in mitochondrial preparations has been most commonly performed by kinetic assays of superoxide dismutase-sensitive epinephrine reduction to adrenochrome or reduction of acetylated or succinylated cytochrome *c* (Boveris *et al.*, 1976; Lass and Sohal, 2000; Nohl and Jordan, 1986; Takeshige and Minakami, 1979; Veitch and Hue, 1994). These assays can be performed with basic equipment and have been applied to submitochondrial particles or to isolated Complex I (Takeshige and Minakami, 1979) and Complex III (Cadenas *et al.*, 1977). The detection of ROS production by functional intact mitochondria needs methods which measure H_2O_2 rather than $O_2^{\cdot -}$. A classic method for the measurement of the rate of mitochondrial H_2O_2 production (Boveris *et al.*, 1972; Boveris and Chance, 1973) used double wavelength spectrophotometry to follow the enzyme–substrate complex between H_2O_2 and cytochrome *c* peroxidase. The method has been applied to heart mitochondria (Boveris *et al.*, 1976). But cytochrome *c* peroxidase is not commercially available, and its preparation from yeast (Prat *et al.*, 1991) would complicate routine assays. Substitution of cytochrome *c* peroxidase by horseradish peroxidase in this assay (Turrens *et al.*, 1985) further decreases the intrinsically low sensitivity of the spectrophotometric ROS detection technique.

A solution is to turn to more sensitive fluorometric techniques, which can be performed also with basic laboratory equipment. One of the earliest ways in which mitochondrial H_2O_2 production was measured was using the scopoletin method (Loschen *et al.*, 1971, 1973). In this assay the fluorescent compound 6-methyl-7-hydroxy-1,2-benzopyrone (scopoletin) is oxidized by H_2O_2 to a nonfluorescent substance in the presence of horseradish peroxidase. While specific, it is a negative method since it is based on the disappearance of fluorescence. In addition to the intrinsic disadvantages of methods that use differences as the basis of estimation, this method requires the use of graded quantities of scopoletin to obtain the best range for measurement (Loschen *et al.*, 1971, 1973). Perhaps the most commonly used fluorometric method is based on the fluorescence derived from oxidation products of the nonfluorescent 2′,7′-dichlorofluorescin (DCF). Nonionic DCF-diacetate is nonpolar, readily crosses cell membranes, and is intracellularly hydrolyzed by esterases-to-nonfluorescent DCF, which is rapidly oxidized by ROS to the fluorescent product 2′,7′-dichlorofluorescein (Keston and Brandt, 1965; LeBel *et al.*, 1992). This reaction is unspecific for H_2O_2 since DCF can be oxidized also by other cellular oxidants, although not by $O_2^{\cdot -}$ (LeBel *et al.*, 1992). Intracellular fluorescence due to DCF oxidation has been considered either to be inversely related to antioxidant levels (van Reyk *et al.*, 2001) or to represent intracellular free radical "production" (Kane *et al.*, 1993). But any antioxidant present between the free radical generator and DCF inside the cells will decrease the final fluorescence. Thus, the final values will indicate the free radical concentration or the overall degree of intracellular oxidative stress, not only free radical production. DCF is commonly used as an estimator of oxidative stress inside isolated cells (Dawson *et al.*, 1993; Driver *et al.*, 2000; García-Ruiz *et al.*, 1995; Kane *et al.*, 1993; Reynolds and Hastings, 1995; Wolf *et al.*, 1997). DCF-based assays have been occasionally applied also to the measurement of free radical production in isolated mitochondria (García-Ruiz *et al.*, 1995). Nevertheless, the applicability of the DCF method to the measurement of ROS production in isolated mitochondria is seriously limited because the noncatalyzed reaction between DCF and H_2O_2 is a very slow process which requires hours to reach completion. This is why measurements in isolated mitochondria using DCF were performed after 60 or even 125 min of reaction (García-Ruiz *et al.*, 1995). This extremely slow response makes the DCF method inappropriate to study quick effects (in the order of seconds) of respiratory inhibitors, physiological modulators like ADP, or drugs, on the rates of mitochondrial ROS production. For the same reason, the DCF method will be also inappropriate for the study of basal rates of ROS production. Incomplete reaction of DCF with H_2O_2 would lead to an underestimation of H_2O_2 levels. Furthermore, in the case of isolated mitochondria, an appropriate detector of ROS production should stay outside the mitochondria instead of entering them. But DCF-diacetate is a lipophilic compound which readily crosses cellular membranes, including the mitochondrial inner and outer membranes, loading into mitochondria (French *et al.*, 1998; Swift and Sarvazyan, 2000). There, the ester is cleaved by nonspecific esterases, allowing DCF to bind to structures within the mitochondria, and its net negative charge also retards its release from the matrix (French *et al.*, 1988). Like in living cells, any matrix antioxidants situated between the free radical generator at the inner membrane and DCF would decrease the final fluorescence readings. Thus, the measurement would indicate ROS concentration at mitochondria, not ROS production.

This is not the case when the H_2O_2 detector molecules used stay outside mitochondria in the incubation buffer during the assay. Other problems of DCF-based methods relate to the capacity of DCF itself to generate free radicals (Rota *et al.*, 1999) and to inhibit State 3 mitochondrial oxygen consumption (Swift and Sarvazyan, 2000), or to recent data indicating that cytochrome *c* catalyzes dichlorofluorescin oxidation (Burkitt and Wardman, 2001) which can lead to false positive measurements during mitochondrial damage-induced release of cytochrome *c*.

Another fluorescent probe is available for the measurement of H_2O_2 production in isolated mitochondria. That indicator is 4-hydroxy-3-methoxy-phenylacetic acid (homovanillic acid). Enzymatic oxidation of this kind of compound by H_2O_2 has been used to specifically detect H_2O_2 production in isolated mitochondria from a wide variety of tissues and animal species (Barja, 1999; Barja *et al.*, 1994; Esposito *et al.*, 1999; Gredilla *et al.*, 2001a,b; Hansford *et al.*, 1997; Herrero and Barja, 1997a,b; Ku *et al.*, 1993; Poderoso *et al.*, 1996; Sohal *et al.*, 1990). The method, described below in detail, is specific for H_2O_2 because of the presence of horseradish peroxidase and improves a previous description of the procedure (Barja, 1998). Originally designed for measurements in polymorphonuclear leucocytes (Ruch *et al.*, 1983), the method has been adapted to the measurement of H_2O_2 production in intact mitochondria respiring with substrate alone (Barja *et al.*, 1994; Barja and Herrero, 1998; Herrero and Barja, 1997a,b). A variant of this method omitting the presence of the fluorescent probe and the peroxidase during the assay shows decreased sensitivity because of the instability of H_2O_2 micromolar concentrations (Staniek and Nohl, 1999). When appropriately performed, the method can be used to quantify basal and quick changes in the rate of mitochondrial ROS production, as well as to localize the main sites of oxygen radical generation in the respiratory chain, without altering the respiratory control ratio. It can be applied also to potentially altered mitochondria coming from tissues of individuals affected by degenerative diseases or equivalent animal models.

ISOLATION OF MITOCHONDRIA

Tightly coupled intact mitochondria can only be isolated from fresh tissues. To obtain intact mitochondria for the assay of H_2O_2 production, they should be isolated as quickly as possible and from fresh tissue. Many different protocols are available for isolating functional mitochondria. Possible choices are those of Mela and Seitz (1979) for heart and of Lai and Clark (1979) for brain mitochondria. Just after their isolation, the amount of mitochondrial protein is measured, and mitochondria can be maintained in concentrated solution over ice without losing their functional properties during a few hours. As soon as possible after isolation, H_2O_2 production and oxygen consumption are measured by fluorometry and polarography respectively. Measurement of mitochondrial oxygen consumption in a Clark-type electrode without (State 4) and with (State 3) saturant ADP allows calculation of the respiratory control index (RCI) (State 3/State 4 oxygen consumption) as an indicator of the degree of coupling and metabolic activity of the mitochondrial preparations.

FLUORESCENT ASSAY OF MITOCHONDRIAL H_2O_2 GENERATION

Mitochondrial H_2O_2 production is kinetically measured following reaction of H_2O_2 with homovanillic acid in the presence of horseradish peroxidase to form a dimer fluorescent at 312 nm excitation and 420 nm emission (Fig. 1). The incubation medium contains 145 mM KCl, 30 mM Hepes, 5 mM KH_2PO_4, 3 mM $MgCl_2$, 0.1 mM EGTA, and 0.1% fatty-acid-free albumin at pH 7.4. During the preparation of the incubation medium, the pH must be adjusted at the same temperature used during the assay (37°C). The following solutions are prepared in that incubation medium without albumin: 70 U/mL of high purity horseradish peroxidase, 4 mM homovanillic acid, pyruvate/malate (125 mM each), and 250 mM succinate (neutralized to pH 7.4). To a standard fluorometric cuvette, add first a large volume of incubation medium and then add small volumes of the reactants in the following order: mitochondria, horseradish peroxidase, homovanillic acid, superoxide dismutase (SOD, optional), and the substrate (pyruvate/malate, or succinate + rotenone) to start the reaction. The volume of incubation medium added should be around 85% of the total reaction volume (1.5 mL). The volumes added of the rest of the reactants are those needed to reach the following final concentrations: 0.25 mg of mitochondrial protein per mL, 6 U/mL of horseradish peroxidase, 0.1 mM homovanillic acid, 50 U/mL of SOD (optional), 5 mM succinate + 2 μM rotenone, or 2.5 mM pyruvate/2.5 mM malate. At those concentrations, H_2O_2 production is not substrate-dependent. It has been described that H_2O_2 generation starts to decrease strongly at concentrations of succinate below 1 mM (Hansford *et al.*, 1997). In the absence of SOD, the rates represent H_2O_2 production. SOD added in excess converts $O_2^{\cdot -}$ produced (if any) to H_2O_2. Thus, in the presence of SOD, the assay

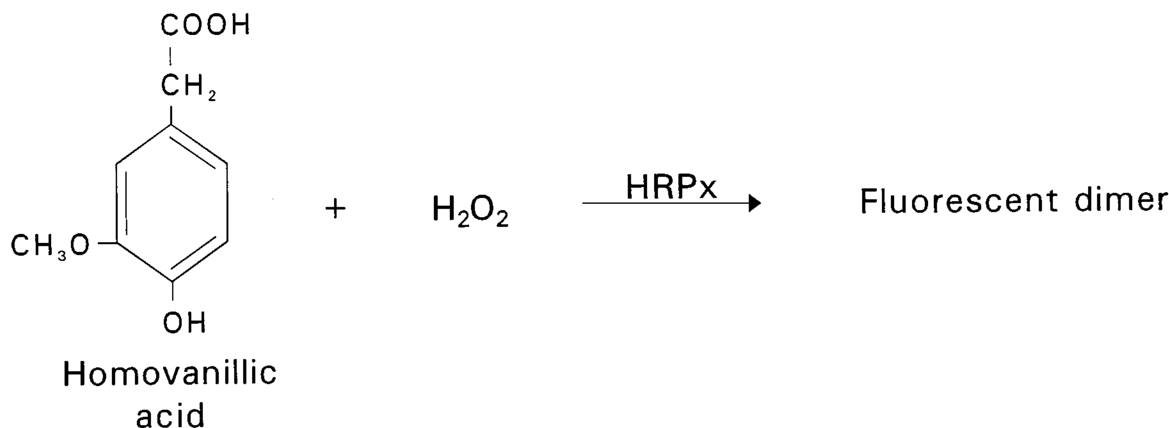

Fig. 1. Homovanillic acid is oxidized by H_2O_2 in the presence of horseradish peroxidase (HRPx) generating a fluorescent dimer. This reaction is used for the sensitive detection of the H_2O_2 produced by isolated mitochondria and liberated to the incubation medium. The presence of HRPx in the medium makes the reaction specific for H_2O_2.

estimates the mitochondrial production of $O_2^{\cdot -}$ plus H_2O_2. In our experience, rat heart mitochondria under State 4 conditions show the same rate of H_2O_2 production with and without SOD. This means that they only secrete H_2O_2 to the outside, not $O_2^{\cdot -}$, but a different situation can be present in other preparations or conditions. In any case, since the respiratory chain univalently reduces oxygen to $O_2^{\cdot -}$ which then dismutates to H_2O_2, the measurement of mitochondrial H_2O_2 production always represents oxygen radical generation.

The reaction is performed during 15 min with constant agitation in a temperature-controlled water bath at 37°C. After 15 min of incubation, the reaction is stopped transferring the samples to an ice-cold bath, 0.5 mL of 0.1 M glycine-NaOH (pH 12) containing 25 mM EDTA are added (per each 1.5 mL of reaction volume), and the fluorescence (312 nm excitation, 420 nm emission) is measured. With the addition of the glycine-NaOH-EDTA the final pH is around 10, which increases the sensitivity and makes the final fluorescence essentially pH-independent (Ruch *et al.*, 1983). Appropriate blanks are also run to correct for the positive fluorescence of the mitochondria themselves. These blanks have mitochondria and all the reaction components but do not contain substrate. Values are obtained by subtracting the fluorescence of the blanks from the fluorescence of the samples and dividing the results by 15 min of incubation. Special blanks (also without substrate) must be included to correct for the fluorescence of antimycin A, thenoyltrifluoroacetone, or myxothiazol, when these mitochondrial respiratory inhibitors are included in the reaction. H_2O_2 secreted to the outside of mitochondria reacts only with the H_2O_2-detection system, since there are no antioxidants in the incubation medium. Addition of external pulses of H_2O_2 during the assay showed that the response of the detection system to the peroxide is instantaneous. They also showed that the amount of H_2O_2 externally added and the amount found with this method are similar.

The arbitrary fluorescence units must be converted to amounts of H_2O_2. For this purpose, some authors use the increase in fluorescence after addition of known amounts of H_2O_2 in the presence of horseradish peroxidase and homovanillic acid. However, if this is selected as a standard, it should be remembered that μM H_2O_2 solutions are unstable. Thus, they should be prepared just before use from stable mM H_2O_2 solutions. A preferred alternative is to use a glucose–glucose oxidase system as a standard. In the presence of excess glucose, this couple generates H_2O_2 at a rate dependent on the amount of glucose oxidase added. For this purpose the following are added to standard tubes: incubation medium, 6 U/mL of horseradish peroxidase, 0.1 mM homovanillic acid, glucose oxidase, and 14 mM glucose (total volume 1.5 mL). The standards are incubated in parallel in the same conditions as the samples (15 min at 37°C, transfer to the ice-cold bath, and addition of 0.5 mL of 0.1 M glycine-NaOH-pH 12 with 25 mM EDTA). Glucose oxidase is added in amounts generating 1 nmol of H_2O_2/min and the fluorescence of samples and standards are compared. This is used to calculate the final mitochondrial production of oxygen radicals, which is expressed in nanomoles of H_2O_2/min·mg of protein. When using this kind of standard, care should be taken that no limiting losses of activity have occurred during transport to or storage of glucose oxidase and horseradish peroxidase at the laboratory.

COMMENTS

Mitochondrial and standard reactions must be performed at controlled temperature and with continuous agitation to ensure appropriate mixing of the reactants and the mitochondria. Control of the pH of the incubation medium is also needed, since the sensitivity decreases below pH 7.4. It is convenient to check the reactants each day before starting the measurements with mitochondria, or even before sacrificing the animal to isolate them. This can be done by chemical (without mitochondria) H_2O_2 pulse experiments, adding H_2O_2 from standard solutions to the homovanillic–horseradish peroxidase detection system and checking the increase in fluorescence. An alternative to this would be to run a standard with glucose–glucose oxidase–horseradish peroxidase and homovanillic acid, although in this case the change in fluorescence will be also dependent on the activity of the glucose–glucose oxidase pair. Those standard reactions are useful to check the quality of the reactants in order to avoid erroneously attributing an absence or very low rate of ROS production to the mitochondrial preparations if a recently received or stored reactant is not in good condition or has lost enzymatic activity.

MITOCHONDRIAL FREE RADICAL LEAK

It is commonly assumed that mitochondrial ROS production is a direct function of mitochondrial oxygen consumption. While this is sometimes true (Ku *et al.*, 1993), in other situations, including comparisons between different animal species (Barja *et al.*, 1994; Barja and Herrero, 1998; Herrero and Barja, 1997b) and between different mitochondrial metabolic states (Herrero and Barja, 1997a), this is not the case. An extreme example is the energy transition from State 4 (nonphosphorylating) to State 3 (phosphorylating) in which mitochondrial oxygen consumption increases acutely, whereas succinate-supported H_2O_2 production is essentially stopped (Boveris *et al.*, 1972; Herrero and Barja, 1997a; Loschen *et al.*, 1971). Another example is hyperthyroidism, in which State 4 oxygen consumption increases but H_2O_2 production does not change in rat heart mitochondria (López-Torres *et al.*, 2000). Thus, the rate of generation of ROS at mitochondria can vary depending not only on the rate of oxygen consumption, but also on the *free radical leak*: the fraction (%) of electrons out of sequence which reduce oxygen to oxygen radicals in the mitochondrial respiratory chain instead of reducing oxygen to water at cytochrome oxidase. The decreases in mitochondrial free radical leak during the State 4 to State 3 transition and during hyperthyroidism allow strong increases in mitochondrial oxygen consumption (and in ATP generation during exercise), without the occurrence of massive increases in free radical production which would damage mitochondria and tissues. Those decreases in free radical leak can be considered adaptive safety devices important to maintain oxidative stress homeostasis at different levels of mitochondrial activity. If mitochondrial H_2O_2 production and oxygen consumption are measured in parallel using the same incubation medium, temperature, and concentrations of substrates and modulators in each mitochondrial preparation, the free radical leak can be calculated. 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 free radical leak is easily calculated by dividing the rate of ROS production by 2 times the rate of oxygen consumption, the result being multiplied by 100. Calculated in this way, the free radical leak (%) of heart mitochondria respiring with succinate in State 4 was 1.3 ± 0.23 in rats and 0.62 ± 0.09 in pigeons, whereas in State 3 it was depressed to around 0.1% of total electron flow (Herrero and Barja, 1997a).

MITOCHONDRIAL SITES OF H_2O_2 PRODUCTION AND CALORIC RESTRICTION

The sites of oxygen radical generation in functional mitochondria can be localized by measuring the rate of H_2O_2 production with a method showing instantaneous response to the inorganic peroxide, and adding different combinations of substrates and inhibitors specific for distinct segments of the respiratory chain. The method used must be sensitive enough to detect oxygen radical production in the absence of respiratory inhibitors and must be specific for H_2O_2. Since the method described in this report fulfils those criteria, it has been used at my laboratory to localize the mitochondrial sites of H_2O_2 generation using inhibitor concentrations having respiratory effects on the mitochondria without perturbing the horseradish peroxidase–homovanillic H_2O_2 detection system. It is well known that the rate of oxygen radical production increases as a function of the degree of reduction of the autoxidizable electron carriers (Boveris and Chance, 1973). Blocking the respiratory chain with an inhibitor increases the reduction state of electron carriers on the substrate side of the inhibitor whereas those in the oxygen (opposite) side change to a more oxidized state. Thus, an increase in oxygen radical production following the addition of an inhibitor means that the oxygen radical generation site is located on the substrate side. Conversely, if oxygen radical production decreases after

addition of the inhibitor, the generator must be situated on the oxygen side. Thus, the higher H_2O_2 production observed in the presence than in the absence of rotenone in pyruvate/malate-supplemented rat mitochondria indicates that they produce ROS at Complex I since this is the only complex situated on the substrate side of the inhibitor in this experiment (Herrero and Barja, 1997b). Similarly, the classically described increase in H_2O_2 production after addition of antimycin A to succinate-supplemented rat heart mitochondria is due to their capacity to produce ROS at Complex III (Boveris *et al.*, 1976; Cadenas *et al.*, 1977). On the other hand, in contrast with the experiment with pyruvate/malate, addition of rotenone to mitochondria respiring with succinate decreases H_2O_2 production, indicating that part of the ROS generated with this substrate comes from Complex I. In this experiment, rotenone blocks the reverse electron flow from succinate to Complex I and thus its capacity for ROS production.

Similar experiments can be used to localize the sites and mechanisms involved in the changes in ROS generation induced by experimental manipulations in animals. A recent example is caloric restriction (Gredilla *et al.*, 2001b). Long-term restriction of food intake decreases H_2O_2 production of rat heart mitochondria with pyruvate/malate as substrates (Table I). However, this does not take place when succinate (plus rotenone) instead of pyruvate/malate is used as substrate (Table I). With pyruvate/malate electrons flow in the respiratory chain through Complexes I and III, whereas with succinate (plus rotenone) they do it through Complexes II and III. On the other hand, it is known that heart mitochondria produce ROS only at Complexes I and III (Boveris *et al.*, 1976; Herrero and Barja, 1997a,b). Thus, the decrease in ROS production induced by caloric restriction in rat heart mitochondria occurs at the Complex I generator, not at Complex III. Additional experiments also clarified the mechanism responsible for the decrease in ROS production induced by caloric restriction. Addition of rotenone to pyruvate/malate-supplemented mitochondria, in agreement with previous experiments (Herrero and Barja, 1997b), strongly increased H_2O_2 generation both in control and caloric restricted rats (Table I), indicating the capacity of Complex I to generate free radicals. However, the difference in ROS production between control and restricted animals, observed with pyruvate/malate alone, disappeared after the addition of rotenone (Table I). In the presence of pyruvate/malate alone, Complex I is partially reduced. In contrast, since rotenone blocks the respiratory chain between Complex I and the ubiquinone pool, in the presence of rotenone plus pyruvate/malate, Complex I is fully reduced. Thus, caloric restriction decreases ROS production in rat heart mitochondria by decreasing the degree of reduction of the Complex I ROS generator in normal conditions (substrate alone). Analogous studies with different combinations of respiratory substrates and inhibitors, using the present method of H_2O_2 detection, can be performed to detect the sites of ROS generation and the mechanisms involved in the variations in ROS production induced in animals by many different experimental situations.

Table I. Rate of H_2O_2 Production in Heart Mitochondria of Control and Caloric Restricted Rats Under Different Conditions

	Nanomoles of H_2O_2/min·mg protein	
	Old control	Old restricted
Pyruvate/malate	0.85 ± 0.11 (6)	0.53 ± 0.09^a (7)
Succinate	1.76 ± 0.25 (6)	1.66 ± 0.26 (7)
Pyruvate/malate + rotenone	8.87 ± 1.2^b (6)	7.4 ± 0.9^b (7)

Note. Old animals had 24 months of age. Caloric restriction was performed during 1 year (Gredilla *et al.*, 2001b). Values are mean ± SEM for the number of animals in parentheses.

[a] Significant difference between food restricted and ad libitum fed control animals.

[b] Significant difference in relation to pyruvate/malate alone in the same group of animals.

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