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The Contribution of Mitochondrial Respiratory Complexes to the Production of Reactive Oxygen Species[1]

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This work was focused on distinguishing the contribution of mitochondrial redox complexes to the production of reactive oxygen species (ROS) during cellular respiration. We were able to accurately measure, for the first time, the basal production of ROS under uncoupled conditions by using a very sensitive method, based on the fluorescent probe dichlorodihydrofluorescein diacetate. The method also enabled the detection of the ROS generated by the oxidation of the endogenous substrates in the mitochondrial preparations and could be applied to both mitochondria and live cells. Contrary to the commonly accepted view that complex III (ubiquinol:cytochrome *c* reductase) is the major contributor to mitochondrial ROS production, we found that complex I (NADH-ubiquinone reductase) and complex II (succinate-ubiquinone reductase) are the predominant generators of ROS during prolonged respiration under uncoupled conditions. Complex II, in particular, appears to contribute to the basal production of ROS in cells.

KEY WORDS: Reactive oxygen species; mitochondria; respiratory inhibitors; ubiquinone.

INTRODUCTION

Reactive oxygen species (ROS) are produced by all eukaryotic cells, predominantly during mitochondrial respiration (Chance *et al.*, 1979). Investigations initiated by Chance and co-workers (Boveris and Chance, 1973; Chance *et al.*, 1979; Loschen *et al.*, 1971) have led to the established view that complex III (ubiquinol:cytochrome *c* reductase or bc_1 complex) and complex I (NADH-ubiquinone reductase) are the major generators of ROS in mitochondria and cells (see for review Boveris, 1984; Turrens, 1997). The evidence sustaining this view has been obtained using methods that often have problems of sensitivity and specificity (Boveris, 1984; Hansford *et al.*, 1997; Korshunov *et al.*, 1997; Turrens and Boveris, 1980; Turrens, 1997), and are not applicable to cellular studies. Moreover, these methods have been used for time courses of a few minutes (cf. Boveris 1984; Loschen *et al.* 1971; Korshunov *et al.* 1997), whereas cellular ROS production is usually measured in time courses of hours (Garcia-Ruiz et al., 1997; Schulze-Osheroff et al., 1992; Zamzami *et al.*, 1995).

Most frequently, ROS production in live cells is measured with dyes that become fluorescent upon reaction with intracellular hydrogen peroxide (Schulze-Osheroff *et al.*, 1992) or superoxide (Zamzami *et al.*, 1995). Studies with these dyes have shown that complex I inhibitors, such as rotenone, decrease ROS production by cells under stress conditions (Garcia-Ruiz et al., 1997; Quillet-Mary *et al.*, 1997; Schulze-Osheroff *et al.*, 1992; Zamzami *et al.*, 1995). While rotenone inhibits the radicals produced by coupled succinate respiration (Hansford *et al.*, 1997; Hin-

[1] Key to abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCF, dichlorofluorescein; DCFDA, dichlorodihydrofluorescein diacetate; DPI, diphenyleneiodonium; ETP_H, electron transfer submitochondrial particles; HRP, horseradish peroxidase; MOA, methoxy-acrylate; Q, ubiquinone; ROS, reactive oxygen species; SOD, superoxide dismutase; TTFA, 2-thenoyltrifluoroacetone.

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kle *et al.*, 1967; Korshunov *et al.*, 1997; Kwong and Sohal, 1998; Loschen *et al.*, 1971), it generally *increases* ROS production in mitochondrial preparations (Boveris, 1984; Kwong and Sohal, 1998; Takeshige and Minakami, 1979; Turrens and Boveris, 1980; Turrens, 1997). Thus, the role of complex I and its inhibitors in mitochondrial ROS production appears to be ambiguous. Given this ambiguity and the possibility that fluorescent probes for ROS detection may induce complex I inhibition (Degli Esposti, 1998), we have developed a very sensitive assay to measure ROS production in both mitochondrial preparations and living cells. This work describes how this assay enables novel evaluations of the contribution of complex I and other respiratory complexes to the mitochondrial production of ROS. The results are especially relevant to the cellular production of ROS over prolonged time periods.

MATERIALS AND METHODS

Materials and Determinations

The ubiquinone (Q) analogue Q-*1* was a generous gift of Eisai Co., Tokyo, Japan. The undecyl-Q analog was the kind gift of Dr. E. Berry (University of California, Berkeley). An average extinction coefficient of 14.5 $mM^{-1}cm^{-1}$ was utilized for determining the concentration of Q analogs (Degli Esposti *et al.*, 1996). Prof. P. Rich (Glynn Laboratory of Bioenergetics, University College of London, UK) provided methoxyacrylate-stilbene (MOA-stilbene), and carboxin was a gift from Dr. J. Hargreaves (AFRC, Long Ashton, UK). Rollinastatin-2 was provided by Prof. D. Cortes (University of Valencia, Spain) and funiculosin was a gift from Dr. P. Bollinger (Sandoz, Switzerland). The concentration of inhibitors was determined spectrophotometrically in absolute ethanol using the reported extinction coefficients (Degli Esposti et al., 1996). Optical and fluorescent probes were purchased from Molecular Probes, Eugene, Oregon, and were determined in methanol as specified by the manufacturer (www.probes.com). Diphenyleneiodonium chloride (DPI) was purchased from Tocris Cookson, (Bristol, UK). Enzymes such as horseradish peroxidase (HRP), *E. coli* superoxide dismutase (SOD) and catalase were from Sigma Co., St. Louis, Missouri. and were dissolved in the same phosphate buffer used for the assay.

Coupled rat liver mitochondria were prepared as described previously (Cain and Skilleter, 1987) but with an additional wash step. Submitochondrial particles (ETP_H) from beef heart were prepared with the procedure of Hansen and Smith (1964), with the following modifications. The particles were also washed with buffer containing 1% BSA and suspended in sucrose 0.25 M, Tris-Cl 0.1 M, pH 7.8, and stored at −70°C. Coupled preparations were prepared as described (Degli Esposti et al., 1996). Protein concentration was determined by the biuret assay (Degli Esposti *et al.*, 1996; Hansen and Smith, 1964). COS-7 cells were cultured at 37°C and 5% CO_2 in Dulbecco's Modified Eagle medium supplemented with 10% (w/v) of fetal calf serum and 2.5% of horse serum and counted microcytometrically. Before the experiments, cells were trypsinized, collected by centrifugation, and resuspended in PBS at $1–2 \times 10^6$/ml for a few hours to exhaust endogenous substrates.

Enzyme Assays

The NADH-Q reductase activity of ETP_H was assayed in 50 mM K-phosphate 1 mM EDTA buffer, pH 7.6, with 100 μM NADH and 50 μM Q-1 or 30 μM undecyl-Q as described previously (Degli Esposti *et al.*, 1996). Succinate dehydrogenase was routinely activated by incubation with 1 mM malonate at room temperature for 30 min. The activity of succinate-ubiquinone reductase was measured by following at 280–400 nm the reduction of 20 μM Q-1 induced by 10 mM K-succinate (Trumpower and Simmons, 1979). Mitochondrial respiration was measured at 30°C with an oxygraph (model 781, Strathkelvin Instruments, Glasgow, UK) under the same conditions as in the ROS assays. The electric membrane potential generated by substrate oxidation was measured at 30°C by following the absorbance changes of 3 μM oxonol VI at 630–601 nm (Degli Esposti *et al.*, 1996) under the same conditions as those used for the ROS assays.

ROS Assays

ROS production by mitochondrial preparations and cells was measured by following the fluorescence increase of the leuko probe dichlorodihydrofluorescein diacetate (DCFDA) (Black and Brandt, 1974; Degli Esposti and McLennan, 1998; Garcia-Ruiz *et al.*, 1995; Hinkle *et al.*, 1967; Nieminen *et al.*, 1997). Contrary to other fluorescent probes, DCFDA did not inhibit the activity of complex I at the concentrations used (Degli Esposti, 1998). Dichlorofluorescein (DCF) flu-

orescence was measured in spectrofluorimeters or with automated detection in fluorescence plate readers (Degli Esposti and McLennan, 1998). Each well of a 96-well Labsystems microtiter plate had phosphate or PBS buffer, pH 7.6, containing 1 μM of DCFDA (diluted from a stock solution in DMSO) and 0.5 mg/ml of ETP_H, or 150–400,000 COS-7 cells to a final volume of 0.15 to 0.2 ml. The reaction was started by either 0.2 mM NADH or 10 mM succinate and usually followed, after 3–5 min required to set the instrument and a cycle of automated shaking, for 1 h, at 30°C in a Molecular Dynamics Biolumin 920 plate reader. The fluorescence readings (excitation at 485 nm and emission at 520 nm, with 5-nm bandwiths) were automatically accumulated every 1 or 2 min. Appropriate controls were run in the same microplate to evaluate blank fluroescence in the presence of inhibitors, either alone or in combination with DCFDA. The controls established that fluorescent inhibitors such as antimycin and MOA-stilbene produced negligible interference with DCFDA fluorescence (cf. Fig. 1).

In some experiments, antibody-conjugated HRP (Silenus, Melbourne, Australia) or Sigma HRP were added to a final dilution corresponding to 0.1–1 units. The external peroxidases enhanced severalfold the rates of DCFDA oxidation by mitochondrial preparations (Table I) without changing the qualitative effect of respiratory inhibitors. The quantitative evaluation of the DCF fluorescence measured by the plate reader was undertaken with a serial dilution of an extensively oxidized DCFDA solution or with DCF (Sigma) (Black and Brandt, 1974). Within the linear range of instrument response, 1 nM DCF corresponded to 3400–4000 arbitrary unit (a.u.) of fluorescence (Degli Esposti and McLennan, 1998).

Table I. Effectors of the Rates of DCFDA Oxidation in Coupled ETP_H[a]

Effector (concentration)	Control rate (%)
A. NADH as the substrate	
Catalase (1000 units)	40
Antimycin (0.5 μM) and catalase	32
CCCP (0.25 μM)	45
B. Succinate as the substrate	
Horseradish peroxidase (0.1 units)	745
Horseradish peroxidase (0.1 units) and catalase (1000 units)	79
Superoxide dismutase (10 units)	131
Mannitol (150 mM)	87
DMSO (40 mM)	90

[a] Control rates were calculated from the linear portion of the time course after 15 to 20 min of reaction (cf. Figs. 2, 3, and 4); their values ranged between 0.3 and 1 pmol/min/mg of protein without effectors. The experimental conditions were as those in Figs. 2, 3, and 4.

RESULTS

Nature of the ROS Detected by DCFDA

As demonstrated by the results reported in Table I, our microplate assay primarily detects hydrogen peroxide among the radicals produced by ETP_H. Incubation with catalase largely decreases the fluorescence signals generated with NADH (Table I), while external peroxidases such as HRP strongly enhance the rates of DCF production (Table I). All previous methods for hydrogen peroxide detection with fluorescent probes have used HRP to enhance sensitivity and have not reported data in the absence of external peroxidases (cf. Loschen *et al.*, 1971; Boveris, 1984; Korshunov *et al.*, 1997). We established that our method is so sensitive that it detects ROS production well above dye background, even without HRP addition, and that results obtained with inhibitor-treated preparations were qualitatively comparable in the absence or presence of external peroxidases. Because peroxidases also increased the spontaneous oxidation of DCFDA without mitochondria (not shown), we decided to omit their addition in most subsequent experiments. SOD slightly stimulated the rates of DCFDA oxidation (Table I), thereby excluding that superoxide anions significantly contribute to the measured signals. Moreover, specific scavengers of hydroxy radicals, such as mannitol and DMSO, marginally decreased the rates of DCFDA oxidation promoted by mitochondrial preparations (Table I).

ROS Production Under Uncoupled Conditions

The membrane potential regulates the rate of ROS production by mitochondria, so that partial uncoupling inhibits the generation of oxygen radicals (Boveris and Chance, 1973; Hansford *et al.*, 1997; Korshunov *et al.*, 1997). We have confirmed this with the DCFDA probe in both coupled mitochondria and submitochondrial particles. Addition of protonophores, such as

CCCP and gramicidin, decreases the rate of DCF production (Table I), even if not so strongly as previously observed with other methods (Boveris and Chance, 1973; Korshunov *et al.*, 1997, Hansford *et al.*, 1997). Possible reasons for this difference include the much longer time course of our measurements and the presence of endogenous anti oxidant enzymes in our mitochondrial preparations. In fact, the addition of the catalase inhibitor aminotriazole did enhance the rate of DCF production in heart preparations (results not shown). Furthermore, previous methods are not sensitive enough for measuring the slow rates of ROS production under uncoupled conditions (Boveris and Chance, 1973; Boveris, 1984; Hansford *et al.*, 1997; Korshunov *et al.*, 1997; Turrens and Boveris, 1980; Turrens, 1997). Consequently, the effect of inhibitors on ROS production has been mainly described in coupled mitochondria and generally with short time courses. Given that with DCFDA the effect of various inhibitors is similar in either coupled or uncoupled conditions (Table I and results not shown), our assay allows the first accurate evaluation of the contribution of respiratory complexes to the prolonged production of ROS in uncoupled mitochondrial preparations.

Role of Complex I in ROS Production

Our microplate assay also enables the first evaluation of the effects of respiratory inhibitors on the production of ROS due to the oxidation of endogenous substrates present in mitochondrial preparations. Figure 1 shows an extended time course of DCFDA fluorescence in ETP_H respiring endogenous substrates, which predominantly feed electrons through complex I, since their respiration is strongly inhibited by rotenone. Concentrations of rotenone allowing maximal inhibition of NADH oxidation (Ramsay and Singer, 1992; Singer 1979; Cadenas *et al.* 1977) decrease the rate and extent of ROS production (Fig. 1A). A similar inhibitory effect is shown by rolliniastatin-2 (Fig. 1A), a potent natural inhibitor of complex I, and by the flavin reagent DPI (Ragan and Bloxham 1977). This decrease in hydrogen peroxide production diminishes by increasing NADH (Fig. 1A, cf. 4B) (see also Kwong and Sohal 1998; Takeshige and Minakami, 1979; Turrens and Boveris, 1980; Turrens, 1997). Moreover, concentrations of rotenone that largely exceed those required for full inhibition of complex I enhance the production of ROS via endogenous substrates (Fig. 1A, top line) or external NADH (not shown).

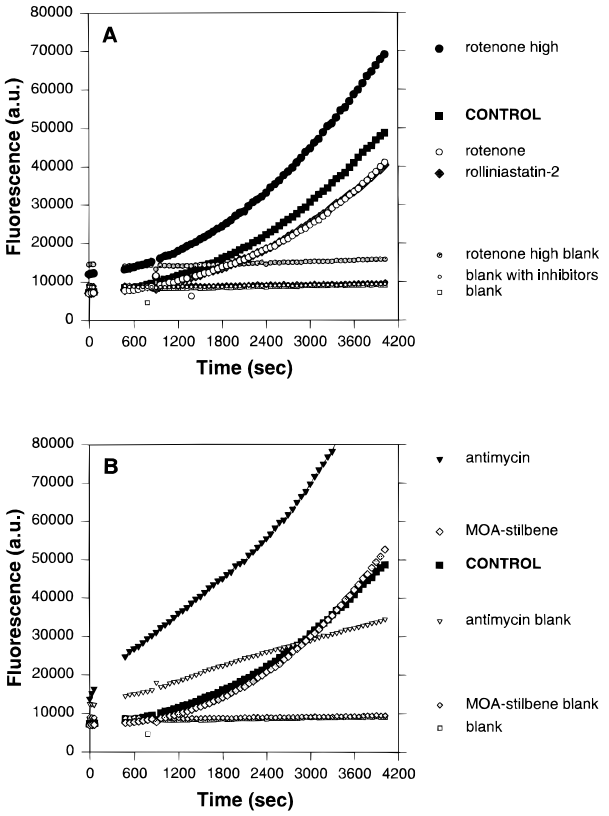

Fig. 1. ROS production with the endogenous substrates in ETP_H. This experiment was conducted in duplicate samples due to the very low level of standard deviation. The measurements were carried out every 60 sec with the particles and then interrupted (gap in the time courses) for ca. 6 min to mix a small volume of buffer in the wells and match the addition of NADH to parallel samples. This was undertaken to allow a direct comparison of DCFDA oxidation with endogenous substrates and external NADH, which resulted in faster rates but diminished effects of complex I inhibitors. (A) The experiment was run for a prolonged time course (over 1 h) to evidence the induction phase in the rate of ROS production in the absence of exogenous substrates. Enzyme assay performed at the end of these experiments confirmed that the mitochondrial particles retain essentially normal activity of electron transport. The effects of complex I inhibitors were measured with 0.5 μM rotenone (1 nmol/mg, open circles) and also 5 μM (rotenone high, filled circles), a concentration comparable to those used in previous works (Garcia-Ruiz *et al.*, 1995; Giulivi *et al.*, 1995; Hennet *et al.*, 1993; Takeshige and Minakami, 1979; Turrens and Boveris, 1980). The concentration of rolliniastatin-2 was 0.25 μM. The blanks with both 0.5 μM rotenone and rolliniastatin-2 were superposable to the control blank (labeled blank with inhibitors), whereas the blank with high rotenone had a higher basal reading, probably reflecting an increased light scattering. (B) Antimycin was used at 0.5 μM and MOA-stilbene at 2 μM to obtain full inhibition of complex III (Ghelli *et al.*, 1997). Note the rapid increase of fluorescence in the antimycin sample even before the interruption. Addition of BSA in the antimycin blank apparently quenched the spurious production of ROS (not shown).

Role of Complex III in ROS Production

Consistent with previous observations (Boveris and Chance, 1973; Boveris, 1984; Chance *et al.*, 1979; Garcia-Ruiz *et al.*, 1995, Giulivi *et al.*, 1995; Hansford *et al.*, 1997; Korshunov *et al.*, 1997; Kwong and Sohal, 1998; Loschen *et al.*, 1971; Takeshige and Minakami, 1979; Turrens and Boveris, 1980; Turrens *et al.*, 1985; Turrens, 1997), antimycin substantially increases the extent and rate of ROS formation in ETP_H, with either endogenous (Fig. 1B) or exogenous substrates (Figs. 2 and 3). However, antimycin blanks apparently show a significant production of ROS in the absence of ETP_H (Fig. 1B). This spurious reaction, which is not related to the weak fluorescence of the inhibitor, has not been noted before (e.g., Garcia-Ruiz *et al.*, 1995, 1997; Turrens and Boveris, 1980) and may derive from a redox-active capacity of the inhibitor, which is unrelated to its specific binding to complex III, as indicated by experiments carried out in the presence of external albumin that removes the unbound inhibitor. Funiculosin, a potent complex III inhibitor acting at the same site as that of antimycin, does not show any comparable effect in blanks with DCFDA (results not shown). Contrary to antimycin, both funiculosin and the specific-center oinhibitor MOA-stilbene (Brandt and Trumpower, 1994) generally have little effect on the production of ROS with endogenous substrates (Fig. 1B). With excess of NADH, MOA-stilbene hardly affects the control rate at short times, but significantly enhances the basal production of ROS at times longer than 30 min (Fig. 2A). In combination with antimycin, MOA-stilbene induces the so-called "double-kill" effect on ROS production that is characteristic of center oinhibitors (Brandt and Trumpower, 1994; Ksenzenko *et al.*, 1983; Rich, 1996; Turrens *et al.*, 1985) (Fig. 2A). However, the effect of MOA-stilbene is less pronounced than that previously reported with the classical center oinhibitor myxothiazol (Dawson *et al.*, 1993; Giulivi *et al.*, 1995; Hansford *et al.*, 1997; Korshunov *et al.*, 1997; Ksenzenko *et al.*, 1983; Turrens *et al.*, 1985). This difference is likely to derive from the fact that myxothiazol, but not MOA-stilbene, inhibits also complex I (Degli Esposti, 1998). In fact, myxothiazol inhibits more effectively the production of ROS associated with NADH oxidation than that due to succinate oxidation (Figs. 2B and 3). Effects similar to those of myxothiazol were observed using stigmatellin, another

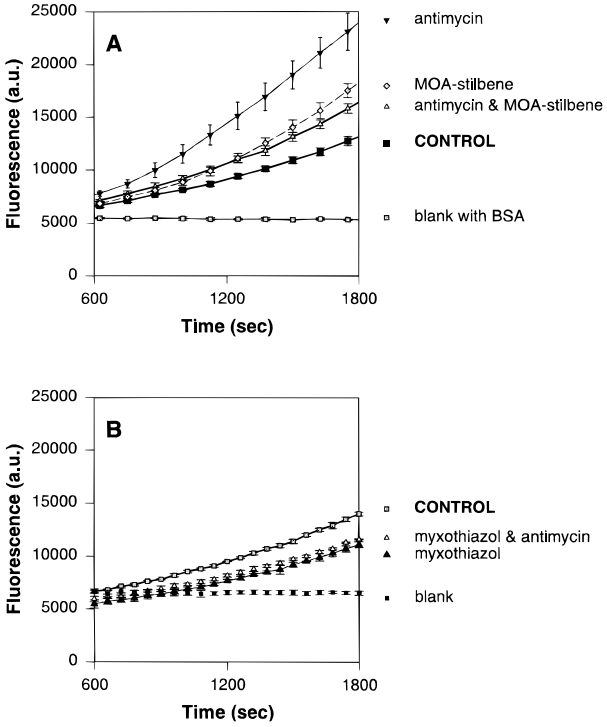

Fig. 2. Complex III inhibitors and the ROS production with NADH. The data were the mean of triplicates +/− S.D. and were obtained after mixing the submitochondrial particles (0.5 mg/ml) with buffer containing the inhibitors (cf. Degli Esposti and McLennan, 1998) to minimize spurious reactions (cf. Fig. 1). The concentration of myxothiazol was 1 μM, i.e. 2 nmol/mg; the other inhibitors were as in Fig. 1. Blanks with either MOA-stilbene or myxothiazol were basically identical to the reference blanks shown in the panels (cf. Fig. 1). (A) The results were obtained with antimycin and MOA-stilbene and the data collected every 120 s. (B). The results of a separate experiment were obtained with antimycin and myxothiazol and the data collected every 60 s. Note that myxothiazol decreases ROS production vs. control, contrary to MOA-stilbene (cf. part A).

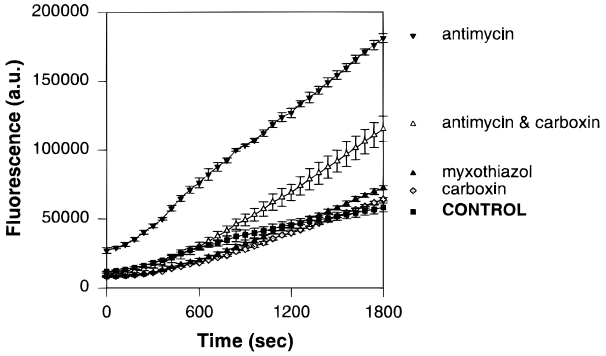

Fig. 3. Inhibition of ROS production with succinate. The experimental conditions were identical to those of Fig. 2B, except that 1 mM malonate was added to the submitochondrial particles to activate complex II. Carboxin was added at 20 μM and induced over 80% inhibition of complex II activity. Succinate concentration was 10 mM.

center oinhibitor which also inhibits complex I like myxothiazol (Degli Esposti *et al.*, 1994).

The Role of Complex II in ROS Production

We next examined the effects of carboxin, the most potent among the classical inhibitors of complex II (Mowery *et al.*, 1976; Keon *et al.*, 1994). Carboxin does not show any spurious reaction with DCFDA and slightly decreases the rate of ROS produced by ETP_H with endogenous substrates (results not shown). At concentrations that specifically inhibit complex II activity by over 80%, with little effect on either complex I or complex III activity, carboxin largely decreases the stimulation of ROS induced by antimycin with succinate (Fig. 3). Although this is not entirely surprising in view of the inhibition of succinate respiration (Mowery *et al.*, 1976), it is noted that a similar effect is not seen with the combination of rotenone and antimycin when NADH is the substrate (results not shown). Hence, inhibition of complex II, but not of complex I, diminishes the ROS production, which is stimulated by antimycin.

Interestingly, concentrations of carboxin that do not affect complex I activity substantially decrease both the rate and extent of ROS produced by NADH respiration (Fig. 4). The effect of carboxin is also seen when a combination of both succinate and NADH are used as substrates, a condition closer to the physiological situation (Boveris and Chance, 1973; Gutman, 1985). Carboxin halves the ROS levels measured with either substrate and essentially abolishes ROS production when it is combined with rotenone (Fig. 4). Carboxin also decreases the production of ROS in coupled rat liver mitochondria respiring on glutamate and malate (results not shown).

Similarly to carboxin, rotenone or rolliniastatin-2 decreases the rate of ROS generation with all substrate conditions (Fig. 4 and results not shown). Conversely to carboxin, the succinate dehydrogenase inhibitor malonate has little effect on the ROS produced by NADH respiration (Fig. 4B, insert). This indicates that a complex II site distal to the succinate oxidation site in the dehydrogenase domain contributes to ROS production stimulated by NADH respiration.

Comparison of ROS Production by Submitochondrial Particles and Cells

Another feature of our method is that it enables a detailed comparison of ROS production in both mitochondrial preparations and living cells (Fig. 5 and Degli Esposti and McLennan, 1998). COS-7 cells have been chosen for this comparison in view of their abundant content of mitochondria and their frequent use in cellular biology (Wolter *et al.*, 1997). Under the conditions of our assays, COS-7 cells show rates of ROS production that are similar to those obtained with NADH-respiring ETP_H in the same microplate (Fig. 5). Whereas antimycin induces a strong stimulation of ROS production in cells similarly to ETP_H, other respiratory inhibitors seem to have little effect on the initial rate of ROS production by live cells in the

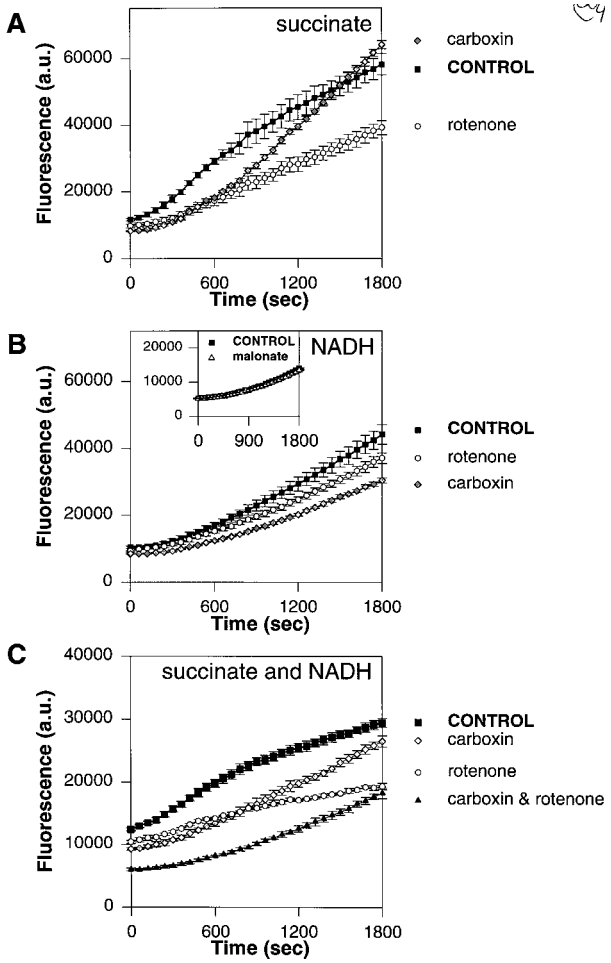

Fig. 4. ROS production by succinate and NADH. The experimental conditions were identical to those of Fig. 3. (A).The particles were supplemented with 10 mM succinate; the concentration of carboxin was 20 μM and that of rotenone the same as in Fig. 1. Rolliniastatin-2 had an effect similar to that of rotenone. (B) The particles were supplemented with 0.2 mM NADH as in Fig. 1. The insert shows the lack of inhibition by 10 mM malonate. TTFA, instead, had an effect similar to that of carboxin (results not shown). (C) The particles were activated with both succinate and NADH.

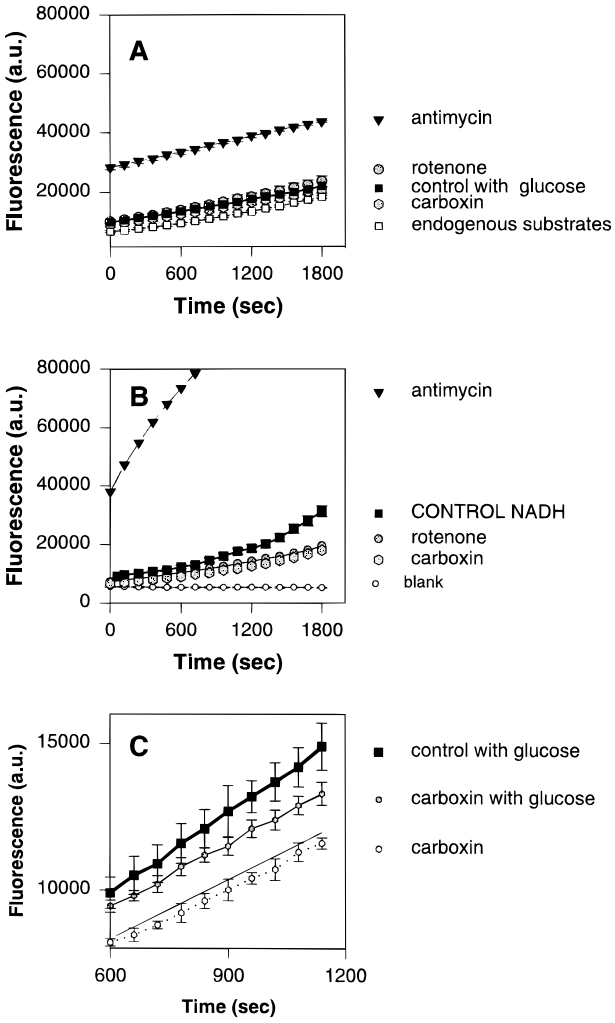

Fig. 5. Comparison of the ROS produced by COS-7 cells and ETP_H. The concentrations of inhibitors were as in Figs. 1–3 and other experimental conditions as described previously (Degli Esposti and McLennan, 1998). (A) COS-7 cells (150,000) were resuspended in PBS ca. 3 h before the experiment in order to exhaust endogenous substrates. All filled symbols refer to triplicate samples supplemented with 20 mM glucose in PBS at time 0. They were all statistically different from the control without glucose (empty squares) in view of the low standard deviations (smaller that the symbols). (B) ETP_H, 0.5 mg/ml, suspended in the same microplate as in (A) and supplemented with 0.2 mM NADH at time 0. The blank did not contain particles or inhibitors. Note that parallel experiments conducted in PBS instead of the phosphate buffer gave very similar results. (C) Results of another experiment in which 400,000 COS-7 cells were assayed with or without 20 mM glucose. The time course of carboxin was significantly lower than the control in the presence of glucose. In the absence of glucose, the time courses with carboxin or other inhibitors were not statistically different from the control (shown as a continuous line for sake of clarity). The standard deviation was larger than in (A) because of the increased light scattering of the more concentrated cell suspension.

absence of nutrients (Fig. 5 and results not shown). Nevertheless, the presence of glucose enhances the production of ROS much more significantly than inhibitors (Fig. 5A,C). Hence, an increased aerobic metabolism modulates the rate of ROS production in cells (Degli Esposti and McLennan, 1998). Within 30 min of incubation, rotenone and other complex I inhibitors tend to decrease the basal production of ROS in the presence of glucose (cf. Degli Esposti and McLennan, 1998). However, with incubations longer than 1 h, rotenone often increases ROS production. This could derive from accumulated high levels of intramitochondrial NADH in glucose-respiring cells that can stimulate autooxidation of low-potential cofactors in complex I. Carboxin, instead, consistently induces a decrease in the basal rate of cellular ROS production when glucose is present in the medium (Fig. 5C). Similar results to those in Fig. 5 have been obtained in other cell lines, such as Daudi lymphoma.

DISCUSSION

This work presents the first accurate documentation of the effects of respiratory inhibitors on the basal production of ROS under uncoupled conditions of electron transport. Previously, tight coupling or respiratory inhibitors were required to allow the detection of ROS during mitochondrial respiration (Boveris and Chance, 1973; Boveris, 1984; Hansford *et al.*, 1997; Loschen *et al.*, 1971; Korshunov *et al.*, 1997; Kwong and Sohal, 1998; Turrens and Boveris, 1980; Turrens, 1997). Because of the qualitative correspondence in the results obtained in coupled and uncoupled preparations, the low rates of ROS production by submitochondrial particles reflect genuine reactions of the mitochondrial respiratory complexes. Our data do not allow an in-depth discussion of the influence of membrane potential on the free radical production of mitochondria, because they are obtained in prolonged time courses and under conditions that do not maximize ROS detection. However, our results reflect situations, which are closely related to those occurring in live cells, where endogenous antioxidant mechanisms prevent rapid large bursts of ROS production as previously detected with isolated mitochondria.

The use of respiratory inhibitors, such as antimycin, has led to the established view that complex III is the major ROS generator in mitochondria (Boveris and Chance, 1973; Boveris, 1984; Garcia-Ruiz *et al.*, 1995; Giulivi *et al.*, 1995; Korshunov *et al.*, 1997;

Kwong and Sohal, 1998; Loschen *et al.*, 1971; Turrens *et al.* 1985; Turrens, 1997). Although our results confirm that antimycin enhances the production of ROS (Figs. 1–3), the interpretation of this and previous evidence needs to account for some new observations made here. Contrary to other inhibitors, antimycin produces a spurious reaction with ROS probes that suggests it may induce a production of oxygen radicals independently of its action on complex III (Fig. 1B). Other potent complex III inhibitors, such as funiculosin and MOA-stilbene, do not stimulate ROS production like antimycin. Furthermore, myxothiazol induces strong inhibition of ROS production (cf. Dawson *et al.*, 1993; Giulivi *et al.*, 1995; Ksenzenko *et al.*, 1983; Turrens *et al.*, 1985) which could be partially due to the inhibition of complex I in addition to that of complex III (Degli Esposti *et al.*, 1994; Degli Esposti, 1998) (Fig. 2B). Therefore, we concur with Hansford *et al.*, (1997) that the contribution of complex III to the normal production of ROS may be smaller than previously thought.

Inhibitors of other respiratory complexes generally depress the basal level of ROS production by submitochondrial particles. In particular, complex I inhibitors decrease ROS production with endogenous substrates (Fig. 1A), NADH and also succinate (Fig. 4). Our results agree with those reported previously using low concentrations of NADH in isolated complex I (Cadenas *et al.*, 1977), in mitochondria with reverse electron transport to NAD^+ (Hinkle *et al.*, 1967); Turrens and Boveris, 1980), and in cellular studies (Degli Esposti and McLennan, 1998; Garcia-Ruiz *et al.*, 1997; Hansford *et al.*, 1997; Quillet-Mary *et al.*, 1997; Schulze-Osheroff *et al.*, 1992; Zamzami *et al.*, 1995). Other reports of a stimulation of ROS production by rotenone (e.g., Turrens and Boveris, 1980) were obtained with either excessive concentrations of the inhibitor, that induce spurious reactions (Fig. 1A, cf. Ramsay and Singer, 1992), or high concentrations of NADH. In the latter case, the low-potential iron-sulfur clusters of complex I are substantially reduced and their autooxidation leads to additional ROS production that masks the specific action of rotenone on bound semiquinone radicals (Degli Esposti, 1998; De Jong *et al.*, 1994; Ingledew and Ohnishi, 1980; Kotlyar *et al.*, 1990; Vinogradov *et al.*, 1995). The effects of Q antagonist inhibitors of complex I with high NADH could mimic situations occurring in cells where mitochondrial respiration is impaired. In these cells, respiratory deficiency leads to an accumulation of NADH in mitochondria, which maintains the low-potential cofactors at unusually high levels of reduction so that they tend to autooxidize more readily with molecular oxygen; this is also due to the block in their natural oxidation pathways. In essence, how complex I inhibition influences cellular ROS production depends on the metabolic state of the mitochondria and the consequent reduction level of their redox cofactors.

Inhibition of complex I by rotenone also diminishes the ROS production by succinate respiration, not only under coupled conditions, as previously observed (Chance *et al.*, 1979; Hansford *et al.*, 1997; Hinkle *et al.*, 1967; Korshunov *et al.*, 1997; Turrens and Boveris, 1980), but also under uncoupled conditions (Fig. 4). Hence, the partial reverse electron transport from complex II to the high-potential components of complex I may be a fundamental reaction in the production of mitochondrial ROS. Electron transport in the Q region of the respiratory chain leads to the formation of stable ubisemiquinones intermediates in complexes I, II, and III (Brandt and Trumpower, 1994; Wolter *et al.*, 1997). The semiquinone bound to complex II (called $Q_s^{\cdot -}$) is the most stable and easily reducible (Ohnishi and Trumpower, 1980). $Q_s^{\cdot -}$ (which is different from the antimycin-sensitive semiquinone of complex III) is further stabilized by antimycin, but destabilized by specific complex II inhibitors, such as thenoyltrifluoroacetone (TTFA) and carboxin (Ackrell *et al.*, 1977; Ohnishi and Trumpower, 1980). We suggest that $Q_s^{\cdot -}$ may be a major contributor to the production of ROS by mitochondrial respiration. In fact, carboxin, like TTFA (Cadenas and Boveris, 1980; Garcia-Ruiz *et al.*, 1997; Schulze-Osheroff *et al.*, 1992), quenches a significant proportion of the ROS production that is enhanced by antimycin (Fig. 3). Further, carboxin substantially reduces the ROS produced by NADH oxidation (Fig. 4). The high redox potential of $Q_s^{\cdot -}$ (Ohnishi and Trumpower, 1980) could easily allow its partial reduction via NADH (Gutman, 1985), even through the leak of electrons flowing in the presence of rotenone (Degli Esposti, 1998). In an analogy with the Q antagonist inhibitors of complex I, the major effect of carboxin is to destabilize $Q_s^{\cdot -}$ and thus reduce its normal reaction with molecular oxygen. However, carboxin also stabilizes the FAD semiquinone (Ackrell *et al.*, 1977), which could directly produce additional ROS or stimulate autooxidation of some iron–sulfur cluster. This would explain why carboxin inhibition of ROS production with succinate is limited to a period of about 20 min (Fig. 4A). The work of Zhang *et al.* (1998) has recently supported the concept that the

low-potential cofactors of complex II contribute to mitochondrial ROS production.

Early reports suggested that succinate dehydrogenase could be implicated in the mitochondrial production of ROS (Boveris and Chance, 1973; Boveris *et al.*, 1976; Cadenas and Boveris, 1980; Degli Esposti *et al.*, 1983; Kmetec and Bueding, 1961; Loschen *et al.*, 1971; Trumpower and Simmons, 1979), but subsequently this suggestion has been dismissed (Boveris, 1984; Hansford *et al.*, 1997; Korshunov *et al.*, 1997; Kwong and Sohal, 1998; Turrens, 1997). Our results indicate that complex II could be a major generator of ROS, not only in mitochondria (Fig. 4), but also in cells (Fig. 5). Of note, a mutation of the iron–sulfur cluster that reduces Q enhances ROS production in a bacterial homologue of complex II (Cecchini *et al.*, 1995) and alterations in complex II activity have been described in mammalian cells undergoing apoptosis (Hennet *et al.*, 1993). More recently, a mutation of the cytochrome *b* gene of complex II has been shown to reduce the lifespan of *C. elegans* due to excessive ROS production (Ishii *et al.*, 1998). By combining this evidence with our results, we conclude that Q radicals in complex I and complex II normally generate most of the reactive oxygen species produced by mitochondrial respiration. The contribution of complex III can become relevant under certain conditions of cellular stress (Degli Esposti and McLennan, 1998).

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