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ORIGINAL PAPER

The ROS production induced by a reverse-electron flux at respiratory-chain complex 1 is hampered by metformin

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Abstract Mitochondrial reactive oxygen species (ROS) production was investigated in mitochondria extracted from liver of rats treated with or without metformin, a mild inhibitor of respiratory chain complex 1 used in type 2 diabetes. A high rate of ROS production, fully suppressed by rotenone, was evidenced in non-phosphorylating mitochondria in the presence of succinate as a single complex 2 substrate. This ROS production was substantially lowered by metformin pretreatment and by any decrease in membrane potential ($\Delta\Psi_m$), redox potential (NADH/NAD), or phosphate potential, as induced by malonate, 2,4-dinitrophenol, or ATP synthesis, respectively. ROS production in the presence of glutamate–malate plus succinate was lower than in the presence of succinate alone, but higher than in the presence of glutamate–malate. Moreover, while rotenone both increased and decreased ROS production at complex 1 depending on forward (glutamate–malate) or reverse (succinate) electron flux, no ROS overproduction was evidenced in the forward direction with metformin. Therefore, we propose that reverse electron flux through complex 1 is an alternative pathway, which leads to a specific metformin-sensitive ROS production.

Keywords Metformin · ROS · Oxidative phosphorylation · Rat liver mitochondria · Rotenone · Malonate · Antimycin · Membrane potential

Abbreviations

$\Delta\Psi_m$	electrical potential difference across the mitochondrial inner membrane
ΔpH_m	pH difference across the mitochondrial inner membrane
DNP	2,4-dinitrophenol
JO_2	oxygen consumption rate
ROS	reactive oxygen species
FFA–BSA	free fatty acid – bovine serum albumin

Introduction

Reactive oxygen species (ROS) production is involved in many physiological or pathological major events (Droge, 2002; Thannickal and Fanburg, 2000). However, the question of its nature and of the location of its production in physiological conditions is an important and still yet not very clear issue. Besides the question of mitochondrial versus extra-mitochondrial ROS production, the exact location at the respiratory chain remains not completely solved. Classically, two production sites are recognized at the respiratory chain: complex 1 and complex 3 (Boveris and Chance, 1973; Kudin et al., 2004), and the production of ROS at these sites is mostly revealed by inhibitor additions, namely rotenone for site 1 and antimycin for site 3 (for review, see Turrens,

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1997). Moreover, ROS production at these sites is mainly related to deleterious events (Kagawa et al., 1999) and its physiological role is still poorly understood, though mitochondrial ROS production has been involved in cardiomyocyte preconditioning (Lebuffe et al., 2003; Vanden Hoek et al., 1998).

A reverse electron flux through the respiratory chain complex 1 has long been recognized (Chance and Hollunger, 1961a,b; Hinkle et al., 1967) and a production of ROS linked to this pathway was also shown (Korshunov et al., 1997; Kushnareva et al., 2002; Kwong and Sohal, 1998; Liu et al., 2002; Votyakova and Reynolds, 2001). However, because of the experimental conditions required to demonstrate this ROS production (i.e. isolated mitochondria with lowered natural antioxidant capacity (Korshunov et al., 1997), its physiological relevance is questioned. Moreover, it has been reported using succinate (as single complex 2 respiratory substrate), while in physiological conditions electron supply occurs simultaneously at both respiratory chain complexes 1 and 2. Nevertheless, respiratory chain complex 1 appears to be crucial for the regulation of mitochondrion-related ROS production, possibly at both forward and reverse electron fluxes (Kudin et al., 2004; Vinogradov and Grivennikova, 2005). Interestingly, this specific physiological feature of complex 1 could be linked to the involvement of mitochondrial metabolism in the commitment to cellular death (Bernardi et al., 2001; Desagher and Martinou, 2000; Lee and Wei, 2000; Newmeyer and Ferguson-Miller, 2003) since we have showed that inhibition of complex 1 prevents oxidative-stress-induced cellular death (Chauvin et al., 2001; Detaille et al., 2005; Guigas et al., 2004).

In recent years, it has been reported that metformin, an anti-hyperglycemic agent currently used in the treatment of type 2 diabetes, is a mild inhibitor of the respiratory chain complex 1 (El-Mir et al., 2000). Therefore, the purpose of the present study was to investigate the effect of metformin on ROS production in complex 1. Although we confirm here that metformin pretreatment led to a mild inhibition of complex 1, as compared to rotenone, we found that it also induced a very slight uncoupling effect and powerful inhibition of the ROS production linked to the reverse electron flux.

Materials and methods

Mitochondria preparation

Mitochondria were prepared from livers of fasted Wistar rats, previously perfused for 20 min with Krebs bicarbonate buffer (120 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 24 mM $NaHCO_3$, 1.3 mM $CaCl_2$, pH 7.4) saturated with a mixture of O_2/CO_2 (19:1 by volume) containing or not 10^{-2} M metformin (El-Mir et al., 2000). Immediately after perfusion, livers were rinsed twice in ice-cold extraction medium and mitochondria were prepared according to the standard differential centrifugation procedure in a medium containing 250 mM sucrose, 20 mM Tris–HCl (pH 7.4) and 1 mM EGTA; final resuspension was achieved in the same medium except for EGTA (0.1 mM) (Klingenberg and Slenczka, 1959). The measurement of mitochondrial proteins was achieved with bicinchoninic acid assay (Hill and Straka, 1988). The mitochondrial suspension was stored at 4°C with 1% FFA–BSA.

Fluorescent measurements

Mitochondria (0.5 mg prot/mL) were incubated in various media as indicated in the figure legends. $\Delta\Psi_m$ was measured fluorimetrically (excitation: 498 nm; emission: 524 nm) in the presence of 0.2 μM rhodamine 123 as described (Fontaine et al., 1998). ROS production was assessed by monitoring H_2O_2-induced fluorescence (excitation: 319 nm; emission: 420 nm) of homovalinic acid (100 μM) in the presence of horseradish peroxidase (20 IU) (Ruch et al., 1983) or by monitoring H_2O_2-induced fluorescence of 1 μM Amplex Red (excitation: 560 nm; emission: 584 nm) in the presence of horseradish peroxidase (10 IU) (Votyakova and Reynolds, 2001). In these experimental conditions, the measured ROS production is the sum of the ROS production by the respiratory chain at the two putative sites: complex 1 and complex 3 (see Fig. 1). With glutamate–malate as respiratory substrate, this production is linked to the forward electron flux at complexes 1 and 3. In such condition, the addition of rotenone will exacerbate the production at complex and site, whereas addition of antimycin will increase that at the level of complex 3. With succinate as substrate, the ROS production is augmented by that related to the reverse electron flux at the level of complex 1, which can be estimated from its inhibition by rotenone. With both glutamate–malate and succinate all these putative sites are potentially implicated. In these latter conditions, addition of rotenone has two potential effects at the level of complex 1: increase ROS production linked to the forward flux and decrease that linked to the reverse electron flux. Calibration of H_2O_2 production was obtained by the addition of a known amount of H_2O_2. In prolonged experiments (Figs. 4–6), H_2O_2 production was assessed as the percentage of the proper control, which slightly decreases with time (data not shown). The level of mitochondrial NADH was monitored by recording its relative fluorescence intensity (excitation: 340 nm; emission: 450 nm). Steady-state ADP phosphorylation was achieved in a regenerating system containing 10 mM glucose, 125 μM ATP and increasing concentrations of hexokinase (Nogueira et al., 2001).

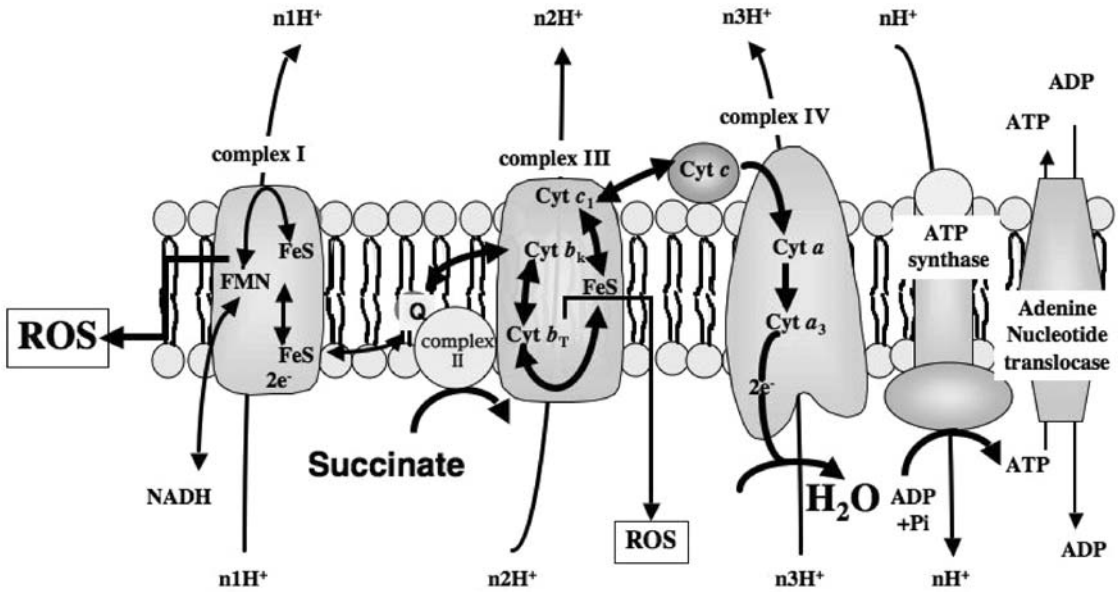

Fig. 1 Schematic view of electron transfer through respiratory chain and ROS production with succinate as single electron donor. When succinate is oxidized by succinodehydrogenase, two electrons are first transferred to the quinone pool, leading to reduced quinone, and then transferred one by one to complex 3. During this process, the transitory formation of semiquinone may lead to the formation of superoxide ion. Electrons are downstream flow until oxygen providing a protonmotive force, leading to ATP synthesis. From the current knowledge, it appears that all the steps involved in electron transfer at the level of respiratory chain are reversible with the notable exception of cytochrome oxidase. Hence, complex 1 electron transfer is also reversible, and with succinate as single respiratory substrate, the proton motive force generated by the downstream electron flux allows a simultaneous reversible flux through complex 1 permitting the reduction of NAD to NADH. Therefore, at complex 1 both forward and reverse electron fluxes are linked to superoxide formation

Fluorimetric assays were performed at 30°C with a double beam PTI Quantamaster C61 fluorimeter, slits widths were set at 4 nm for excitation while they were set for emission at 2 nm for $\Delta\Psi_m$ and 4 nm for ROS production and NADH level.

Mitochondrial respiration

Mitochondrial oxygen consumption was measured polarographically at 30°C in a high-resolution Oroboros® oxygraph system.

Oxidative phsophorylation

To obtain steady states low rate of ATP synthesis by oxidative phosphorylation, we have used a steady-state ADP-regenerating system with ATP–glucose–hexokinase. Addition of successive and low amounts of hexokinase allows steady states rates of ADP generation (as assessed by glucose 6-phosphate accumulation) and ATP synthesis (by oxidative phosphorylation) at constant ATP plus ADP concentration. Glucose 6-phosphate accumulation and oxygen consumption rate allows simultaneous determination of both oxidation and phosphorylation at low nonsaturating ADP concentration.

Reagents

Metformin was a gift from Merck. The bicinchoninic acid assay of protein was from Pierce, Amplex Red was obtained from Molecular Probes, and all other chemicals were purchased from Sigma.

Results

ROS production is associated to a reverse electron transfer at complex 1

As shown in Fig. 2, a single addition of succinate to control rat liver mitochondria induced a large increase in both membrane potential (panel C) and NADH (panel B), and led to a substantial rate of ROS production (panel A), which was almost fully abolished by rotenone addition. This confirms previous findings obtained in isolated brain or heart mitochondria depleted from their natural antioxidant capacity (Liu et al., 2002), contrarily to the present experimental conditions. As expected, the addition of antimycin reactivated ROS formation, although to a much lesser extent. When mitochondria were isolated from a liver previously perfused 20 min with 10^{-2} M metformin (Met-mitochondria), ROS production in the presence of succinate was significantly

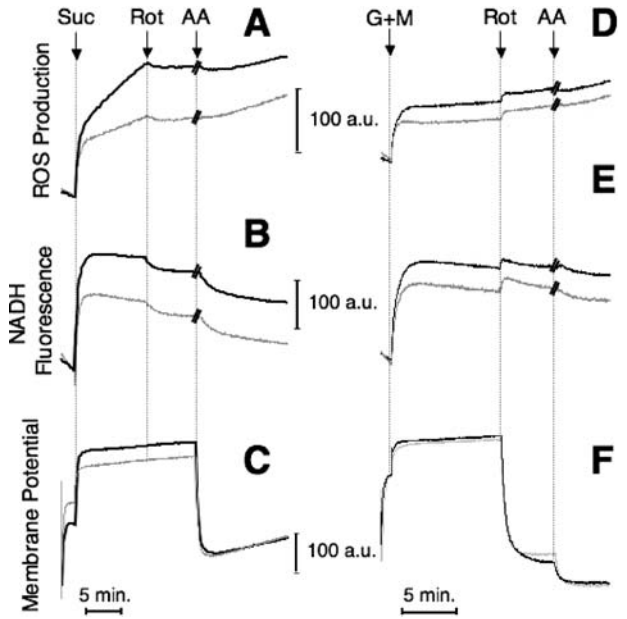

Fig. 2 ROS production, NADH fluorescence, and $\Delta\Psi$ in mitochondria incubated with either succinate or glutamate–malate as respiratory substrates. Mitochondria were isolated from rat liver ex vivo perfused with Krebs-bicarbonate buffer in the presence (*gray line*) or in the absence (*black line*) of 10^{-2} M metformin (see Materials and Methods section) and incubated (1 mg protein) in 2.0 mL medium (30°C) containing 250 mM sucrose, 0.1 mM EGTA, 20 mM Tris–HCl (pH 7.4), 2.5 mM Pi and 1 mM $MgCl_2$. Panels A and D: Hydrogen peroxide (ROS) production was assessed by homovanillic acid/horse radish peroxidase; panels B and E: NADH concentration was followed by determining its spontaneous autofluorescence; panels C and F: $\Delta\Psi$ was determined by rhodamine 123 fluorescence (see Materials and Methods section). Where indicated, substrates and/or inhibitors were sequentially added: 2.5 mM succinate (Suc), panels A–C; 2.5 mM glutamate and 1.25 mM malate (G + M), panels D–F; 1 μM rotenone (Rot) and 0.125 μM antimycin A (AA). Spontaneous fluorescence of antimycin A was subtracted and curves were rescaled after its addition. One typical experiment is presented (see also Table 1)

lower than in control mitochondria (see Fig. 2A and Table 1). On the contrary, the antimycin-related ROS production was not significantly affected by metformin pretreatment (Table 1). As shown in Fig. 2D and in Table 1, ROS production was dramatically lower when mitochondria were energized with glutamate plus malate alone. As expected, NADH level (panel E) and membrane potential (panel F) increased following substrate addition but we did not find any difference within the two groups of mitochondria. Importantly, metformin pretreatment did not affect ROS production in that particular condition, whereas rotenone addition increased ROS production in both groups. Note that such ROS production remained lower than in the presence of succinate before rotenone addition (Table 1).

As shown in Fig. 3A and B, the inhibition of electron transfer by metformin was only partial, as attested by the limited inhibition of the respiratory rate in the presence of glutamate–malate, whereas rotenone was responsible for a nearly complete inhibition of respiration. Of note, the very small respiratory rate observed in the presence of rotenone was sufficient to maintain a low but substantial membrane potential (see Fig. 2F). Antimycin addition further decreased oxygen consumption (Fig. 3A and B) indicating a complete blockage of electron flux through the respiratory chain, which was confirmed by the full collapse of the membrane potential (see Fig. 2F). As shown in Table 2, metformin pretreatment did not significantly affect the respiratory rate in the presence of succinate alone (see Fig. 3C and D). Interestingly, rotenone addition decreased the oxygen consumption in control mitochondria but not in Met-mitochondria, an effect probably related to the inhibition of the ROS-linked oxygen consumption at the reverse electron pathway. As a consequence, the respiratory rate of Met-mitochondria was slightly but significantly increased by 17% (see Table 2), suggesting that metformin pretreatment may slightly uncoupled mitochondria, which in turn may account for the effect of metformin on ROS production.

Because rhodamine 123 was not able to prove any decrease in $\Delta\Psi_m$ in metformin pretreated mitochondria, we next measured the effect of slight concentrations of uncoupler DNP on both oxygen consumption and ROS production. As shown in Fig. 4, the concentration of DNP that decreased H_2O_2 production to the same extent as metformin

Table 1 Effect of metformin pretreatment on H_2O_2 production

	H_2O_2 (pmol/min/mg)								
	Glutamate + Malate			Succinate			Glutamate + Malate + Succinate		
Additions	Control	Metformin	*p* value	Control	Metformin	*p* value	Control	Metformin	*p* value
None	24 ± 7 (3)	23 ± 4 (3)	ns	201 ± 20 (6)	84 ± 13 (6)	<0.01	141 ± 9 (5)	79 ± 4 (5)	<0.01
Rotenone	70 ± 9 (3)	57 ± 12 (3)	ns	3 ± 3 (6)	8 ± 5 (6)	ns	40 ± 21 (5)	38 ± 10 (5)	ns
Rotenone + antimycin	54 ± 5 (3)	48 ± 8 (3)	ns	49 ± 7 (6)	48 ± 9 (6)	ns	64 ± 13 (5)	54 ± 13 (5)	ns

Note. Mitochondria were incubated in the medium described in Fig. 2 supplemented with 2.5 mM glutamate + 1.25 mM malate, 2.5 mM succinate, or 2.5 mM glutamate + 1.25 mM malate + 2.5 mM succinate. H_2O_2 production was measured in the absence or presence of 1 μM rotenone ± 0.125 μM antimycin. Results are mean ± SEM (*n*). Statistical significance was assessed using an unpaired Student's *t* test.

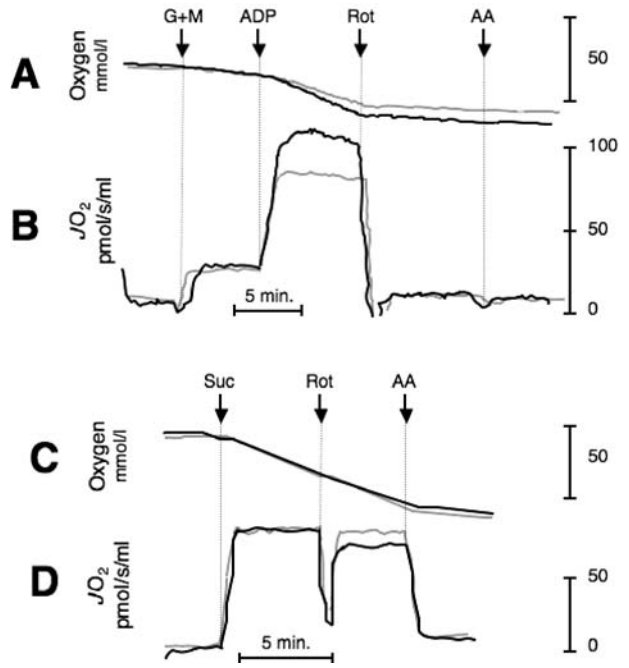

Fig. 3 Effect of metformin pretreatment on oxygen consumption. Mitochondria were isolated as in Fig. 2 from rat liver perfused with buffer containing (*gray line*) or not (*black line*) 10^{-2} M metformin (see Materials and Methods section) and incubated (1 mg protein) in 2.0 mL medium containing 250 mM sucrose, 0.1 mM EGTA, 20 mM Tris–HCl (pH 7.4), 2.5 mM Pi, and 1 mM $MgCl_2$. Where indicated, 2.5 mM glutamate and 1.25 mM malate (G + M), panels A and B; or 2.5 mM succinate (Suc), panels C and D, 0.5 mM ADP, 1 μM rotenone (Rot), and 0.125 μM antimycin A (AA) were added. Oxygen concentration in the medium (panels A and C) and its derivative (panels B and D) was determined using a high-resolution respirometer (Oroboros) at 30°C. One typical experiment is presented and similar results were obtained in three other different preparations

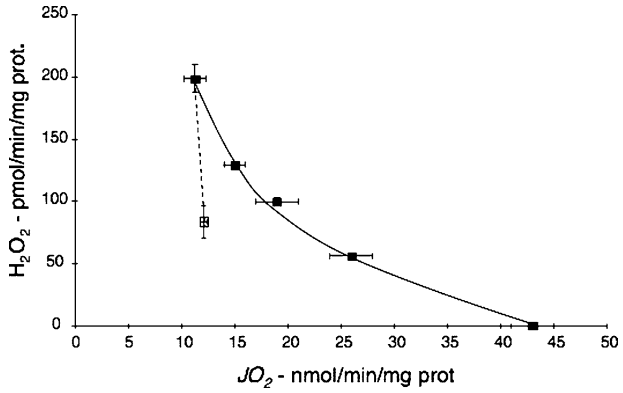

Fig. 4 Relationships between ROS production and oxygen consumption rate. Mitochondria were prepared as described in Fig. 2. Hydrogen peroxide (ROS) production and oxygen consumption were determined in the presence of 2.5 mM succinate as in Figs. 2 and 3, respectively. In control mitochondria (*closed symbols*), oxygen consumption was increased by adding increasing amounts of DNP. *Open symbol* represents ROS production and oxygen consumption of Met-mitochondria in the absence of rotenone. Data represent the mean of at least three different experiments ± SEM

pretreatment increased oxygen consumption by more than 80%. Therefore, the putative uncoupling effect of metformin pretreatment could only account for a limited part of the observed effect on ROS production.

Effect of ΔpH_m on ROS production

Because it has been reported that reverse flux-related ROS production was controlled by ΔpH_m in muscle mitochondria (Lambert and Brand, 2004a,b), we next assessed whether or not this was also the case in liver mitochondria, potentially accounting for the effect of metformin pretreatment. As shown in Fig. 5, abolition of ΔpH_m by nigericin addition increased $\Delta\Psi_m$ as expected (panel B) but did not affect ROS production (panel A) both in normal and in pretreated mitochondria. This observation is not in agreement with the view that ΔpH_m plays a role in the observed effect of metformin on ROS production.

ROS production at complex 1 and reverse electron flux

Because ROS production in the presence of succinate is affected by complex 1 inhibitors (namely, rotenone and metformin), we next investigated the relationship between ROS production and the reverse electron flux through complex 1. Theoretically, such a reverse electron flux depends on the three main parameters: (i) the electron supply downstream of complex 1, (ii) the protonmotive force, and (iii) the span of redox potential across complex 1.

Table 2 Effect of metformin pretreatment on resting respiration

	JO_2 (nmol/min/mg)					
	Succinate			Glutamate + Malate + Succinate		
Addition	Control	Metformin	*p* value	Control	Metformin	*p* value
None	11 ± 0.2 (19)	12 ± .03 (19)	ns	11 ± 0.2 (5)	11 ± 0.1 (5)	ns
Rotenone	9.5 ± 0.2 (14)	11.5 ± 0.3 (14)	<0.001	10 ± 0.3 (3)	10.5 ± 0.3 (3)	ns

Note. Mitochondria were incubated in the medium described in Fig. 3 supplemented with 2.5 mM succinate or 2.5 mM succinate, 2.5 mM glutamate and 1.25 mM malate. Results are mean ± SEM (*n*). Statistical significance was assessed using an unpaired Student's *t* test.

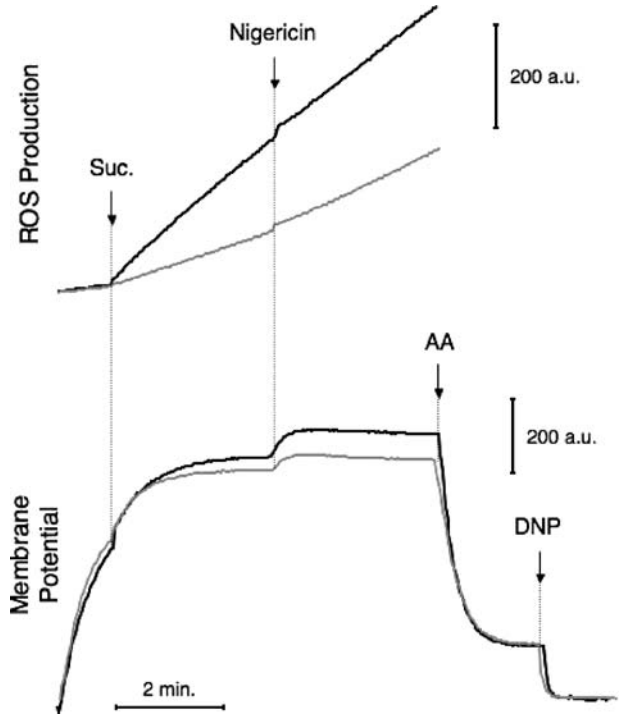

Fig. 5 Effect of nigericin on ROS production and $\Delta\Psi_m$. Mitochondria were incubated at 30°C in a medium containing 125 mM KCl, 1 mM EGTA, 20 mM Tris–HCl (pH 7.4). Hydrogen peroxide (ROS) production of mitochondria (0.5 mg protein) was assessed by Amplex Red/horse radish peroxidase, while $\Delta\Psi_m$ were measured as in Fig. 2. Where indicated, 2.5 mM succinate, 100 nM nigericin, 0.125 μM antimycin, and 75 mM DNP were added. Controls mitochondria: *black line*; Met-mitochondria: *gray line*

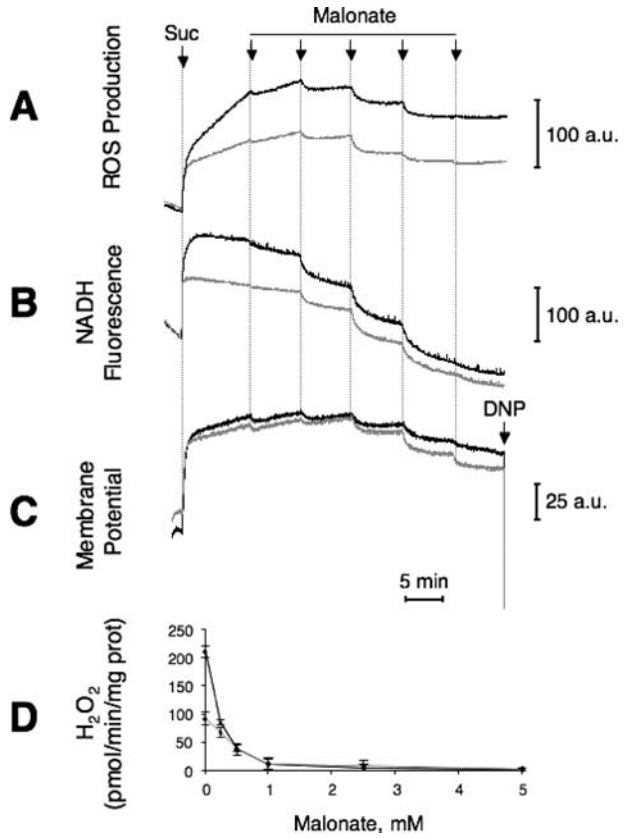

Fig. 6 Effect of malonate on ROS production, NADH fluorescence, and $\Delta\Psi$ in control or Met-mitochondria energized with succinate. Mitochondria were prepared and incubated as described in Fig. 2 (controls: *black line*, Met-mitochondria: *gray line*). Hydrogen peroxide (ROS) production (panel A), NADH concentration (panel B), and $\Delta\Psi_m$ (panel C) were determined as in Fig. 1. Where indicated, 2.5 mM succinate (Suc) and subsequent additions of malonate were performed to reach 0.25 mM, 0.5 mM, 1 mM, 2.5 mM, and 5 mM, respectively (final concentrations). At the end of the experiment, $\Delta\Psi_m$ was fully collapsed by adding 75 μM DNP. Panel D: H_2O_2 production according to malonate concentration; cumulative data of three different experiments ± SEM (see Materials and Methods section for calibration procedure)

Malonate is a well-known inhibitor of both transport and oxidation of succinate, consequently a titration with this inhibitor permits to limit the flux through complex 2 and therefore, the electron supply downstream of complex 1. Figure 6 (panels A and D) shows that the successive additions of small amounts of malonate decreased the production of ROS in a dose-dependent manner in both control and Met-mitochondria. Each addition of malonate resulted in lower steady-state levels of NADH (Fig. 6B) and mitochondrial membrane potential (Fig. 6C).

Mild uncoupling of the respiratory chain by successive additions of small amounts of the protonophore DNP was also responsible for a decreased ROS production at each steady state in both groups of mitochondria (Fig. 7A and D). Both steady-state NADH levels and $\Delta\Psi_m$ decreased (Fig. 7B and C) in a dose-dependent manner.

The effect of ATP synthesis on ROS production was studied in Fig. 8 by adding successive additions of small amounts of hexokinase in the presence of an ADP regenerating system. ROS production was dramatically inhibited after a very small addition of hexokinase (Fig. 8A and D), while NADH level (Fig. 8B) and membrane potential (Fig. 8C) were almost unaffected.

Assuming one single reversible electron pathway through complex 1, the ROS production at complex 1 would depend on the redox status of the production site, which depends on the redox span applied to complex 1 (i.e. NADH/NAD ratio, redox status of the quinone pool, protonmotive force). When glutamate–malate is added to succinate, electron flux through complex 1 in the forward direction could be expected to increase. In this situation, the redox strength applied to the system increases and an increase in the ROS production would be expected. However, as shown in Fig. 9 where succinate and glutamate–malate were sequentially added, ROS production decreased after glutamate–malate addition in control mitochondria and was unaffected in Met-mitochondria (see also Table 1). In both cases, NADH fluorescence and $\Delta\Psi_m$ (Fig. 9B and C) were not affected by the addition of glutamate–malate to succinate.

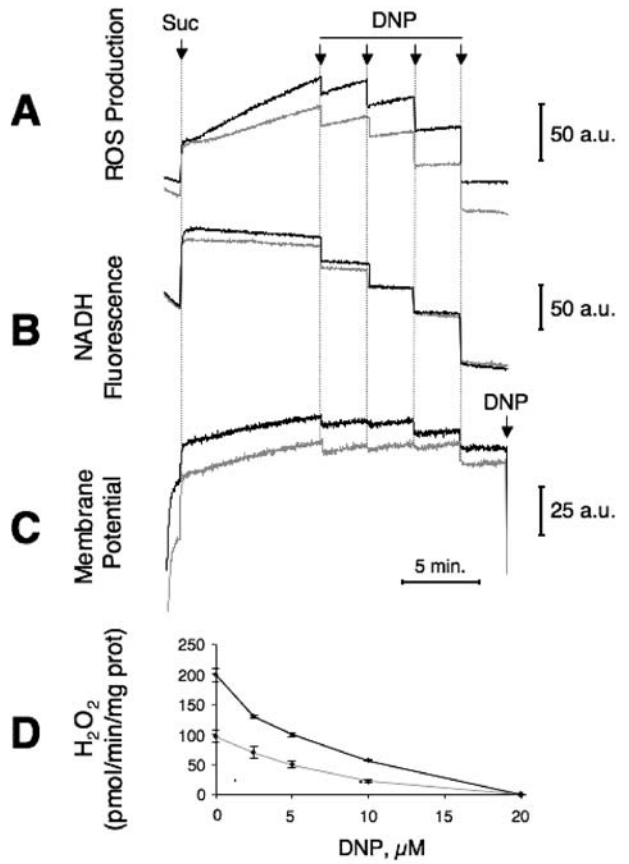

Fig. 7 Effect of uncoupling with DNP on ROS production, NADH fluorescence and $\Delta\Psi_m$ in control or Met-mitochondria energized with succinate. Mitochondria were prepared and incubated as described in Fig. 2 (controls: *black line*, Met-mitochondria: *gray line*). Hydrogen peroxide (ROS) production (panel A), NADH concentration (panel B), and $\Delta\Psi_m$ (panel C) were determined as in Fig. 1. Where indicated, 2.5 mM succinate (Suc) and subsequent additions of DNP were performed to reach 2.5 μM, 5 μM, 10 μM, and 20 μM, respectively (final concentrations). At the end of the experiment, $\Delta\Psi_m$ was fully collapsed by adding 75 μM DNP (final concentration). Panel D: H_2O_2 production according to DNP concentration; cumulative data of three different experiments ± SEM

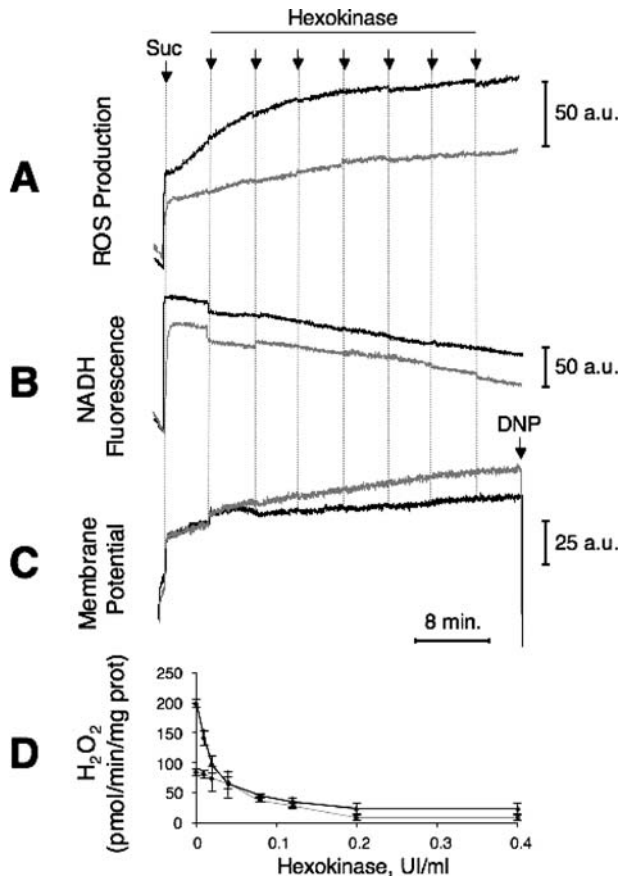

Fig. 8 Effect of ATP synthesis on ROS production, NADH fluorescence and $\Delta\Psi_m$ in control or Met-mitochondria energized with succinate. Mitochondria were prepared and incubated as described in Fig. 2 (controls: *black line*, Met-mitochondria: *gray line*), except that 10 mM glucose and 125 μM ATP were added. Hydrogen peroxide (ROS) production (panel A), NADH concentration (panel B), and $\Delta\Psi_m$ (panel C) were determined as in Fig. 2. Where indicated, 2.5 mM succinate (Suc) and subsequent additions of hexokinase were performed to reach 0.01, 0.02, 0.04, 0.08, 0.12, 0.2, 0.4 IU/mL, respectively (final concentrations). At the end of the experiment, $\Delta\Psi_m$ was fully collapsed by adding 75 μM DNP. Panel D: H_2O_2 production according to hexokinase concentration; cumulative data of three different experiments ± SEM

Discussion

A reverse electron flux through respiratory chain complex 1 has long been described (Chance and Hollunger, 1961a,b; Hinkle et al., 1967). More recently, an ROS production linked to this reverse pathway has been described in several tissues including brain (Liu et al., 2002; Votyakova and Reynolds, 2001), heart (Korshunov et al., 1997, 1998) and muscle (Lambert and Brand, 2004a,b). Assessment of ROS production is difficult since it mostly depends on indirect methods. Moreover, the measurable flux represents only the part of the production escaping the physiological antioxidant protective systems and it is often necessary to decrease the physiological endogenous antioxidant capacity by H_2O_2–aminotriazole pretreatment (Korshunov et al., 1997). It is worth noting that the experiments in rat liver mitochondria presented here have been conducted in the absence of such pretreatment, and we have used a classical method of ROS determination based on the fluorescent determination of hydrogen peroxide (Ruch et al., 1983). From our results it appears that the highest flux of electrons leading to ROS formation is about 1% of total electron flux, i.e. 0.2 nmol H_2O_2/(min mg) (=0.4 nano equivalent electron/(min mg)), whereas the respiratory rate was 11 nmol O_2/(min mg) (=44 nano equivalent electron/(min mg)). In such condition of succinate as unique respiratory substrate and in the absence of rotenone, the consequence of a near-equilibrium state of complex 1 is a high rate of electron recycling through this complex whereas the net flux is low (1% of total electron net flux

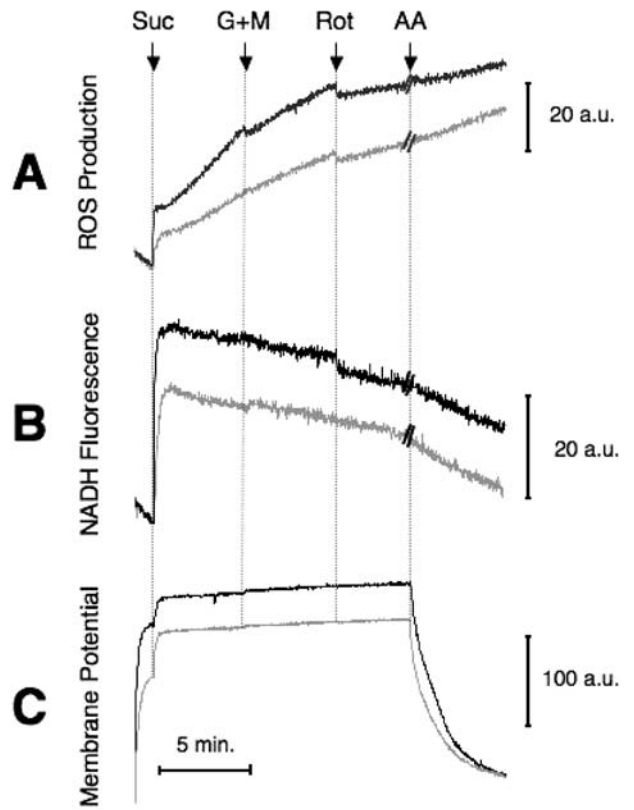

Fig. 9 ROS production, NADH fluorescence, and $\Delta\Psi_m$ in control or Met-mitochondria energized with succinate and glutamate–malate. Mitochondria were prepared and incubated as described in Fig. 2 (controls: *black line*, Met-mitochondria: *gray line*). Hydrogen peroxide (ROS) production (panel A), NADH concentration (panel B), and $\Delta\Psi_m$ (panel C) were determined as in Fig. 1. Where indicated, 2.5 mM succinate (Suc) and 2.5 mM glutamate + 1.25 mM malate (G + M), 1 μM rotenone (Rot), and 0.125 μM antimycin A (AA) were added. Spontaneous fluorescence of antimycin A was subtracted and curves were rescaled after its addition. One typical experiment is presented (see also Table 1)

at most). Moreover, it appears from our results that when considering the different sites for ROS production at the level of the respiratory chain (complex 1 and complex 3), the ROS production linked to the reverse electron flux at complex 1 (succinate alone and rotenone-sensitive) is predominant by far as compared to the other sources linked to forward electron flux since rotenone addition almost fully abolished this ROS production.

This method, which is reported to be sensitive and quite specific, is however sensitive to NADH auto-fluorescence (Batandier et al., 2004). Hence, we have always determined simultaneously NADH auto-fluorescence and ROS production. In addition, the intensity of fluorescence emission could also be affected by a change in mitochondrial volume, however we failed to find a difference between the two groups of mitochondria (data not shown). We found that regardless of the relative changes in NADH fluorescence between each redox steady state, the rate of ROS production (i.e. the slope of the signal) was not linked to a change in NADH because its auto-fluorescence was stable during the considered steady state. Moreover, the decrease in reverse flux ROS production was also observed in metformin pretreated mitochondria by measuring H_2O_2 production with Amplex Red, a fluorescent probe, which is insensitive to NADH autofluorescence (see Fig. 5).

It is classically admitted that the different complexes of the respiratory chain, cytochrome *c* oxidase excepted, work at near equilibrium when the respiratory flux remains low (i.e. close to state 4) (Erecinska and Wilson, 1982; Forman and Wilson, 1982). Our hypothesis was that the reverse flux-related ROS production depends on the redox state of complex 1 constitutive subunits and/or of the magnitude of the reverse flux. Since it is difficult to modulate separately redox state, $\Delta\Psi_m$ and reverse electron flux, we have used several tools to affect these parameters in different ways: inhibitor of complex 2, uncoupler or low rates of phosphorylation. Regardless of the tool, the reverse flux-related ROS production was dramatically inhibited from the smallest addition.

From the data presented here, it appears that the reverse flux-related ROS production is very sensitive to both NADH and $\Delta\Psi_m$ levels. Indeed, if on the one hand the addition of malonate or DNP clearly decreased simultaneously NADH level, $\Delta\Psi_m$ and ROS production, on the other hand hexokinase addition decreased ROS production while NADH and $\Delta\Psi_m$ were almost unchanged. Although it is highly probable that both NADH and $\Delta\Psi_m$ slightly decreased in this condition of moderate induction of ATP synthesis, the experimental tools used to assess these parameters were not sensitive enough to significantly evidence these minor changes.

The protonmotive force is a key parameter in determining the rate of mitochondrial ROS production. Although it has been reported that decreasing ΔpH_m decreases ROS production in muscle mitochondria (Lambert and Brand, 2004a,b) and despite the fact that we confirmed this observation (data not shown), result in Fig. 5 demonstrates that this is not the case in rat liver mitochondria. This may be due to the fact that ΔpH_m is much lower in liver than in muscle, as demonstrated by the small and the large increase in $\Delta\Psi_m$ observed after nigericin addition in liver and muscle, respectively (data not shown). On the other hand, a slight decrease in $\Delta\Psi_m$ has been shown to lead to a dramatic inhibition of ROS (Votyakova and Reynolds, 2001). The different ways used in the present work to influence the reverse electron flux are all known to affect $\Delta\Psi_m$. If complex 1 is close to equilibrium when succinate is oxidized, any change in NADH level reflects both mitochondrial membrane potential and FADH2 levels. Hence, in this specific condition, ROS production in complex 1 depends on the levels of the related potentials NADH, FADH2, and $\Delta\Psi_m$, and any decrease in one (or more) of these forces would decrease ROS production. Such picture is in agreement with the view that ROS

production occurs when a one-electron donor site reaches a high redox level. Addition of glutamate–malate to succinate is then expected to induce a high level of ROS production, since in this particular condition, the related forces are high. However, the results presented here clearly indicate that this is not the case since the rate of ROS production is lower in the presence of glutamate–malate and succinate as compared to succinate alone (compare Figs. 2 and 9, and see Table 1).

Malate oxidation generates oxaloacetate, which in turn might inhibit the succinate dehydrogenase. However, the maximal respiratory rate in the presence of succinate was not affected by the addition of glutamate–malate (data not shown). This demonstrated the absence of any kinetic constraint on succinate oxidation in our conditions, which might have explained the observed inhibition of ROS after glutamate plus malate addition.

If complex 1 works near equilibrium, the decrease in ROS production upon the addition of glutamate–malate to succinate cannot be explained by a decrease in the reverse electron flux. Hence, it seems that the origin of electrons, i.e. upstream or downstream of complex 1, plays a crucial role on the reverse electron flux. This led us to propose, as already hypothesized (Vinogradov and Grivennikova, 2001), the existence of two distinct electron pathways at the complex 1 regarding forward and reverse fluxes, both being coupled to a proton translocation but in opposite directions (Fig. 1). Supporting this proposal, several separate electron pathways in the complex 1 have been evidenced using different classes of inhibitors (Anderson and Trgovcich-Zacok, 1995).

The present results indicate that a rotenone- or metformin-inhibited ROS production linked to reverse electron flow at the complex 1 is not at thermodynamic equilibrium. It is very sensitive to minor change in $\Delta\Psi_m$ as already reported (Korshunov et al., 1997) and regulated by glutamate–malate oxidation. These properties confer a high elasticity of this particular ROS production toward the respiratory chain electron supply at both site 1 and site 2. Such specific ROS production has probably a physiological meaning since it occurs in the presence of a simultaneous electron supply at both site 1 and site 2, as it is the case in living cells, without any inhibitor addition or attempt to lower the natural antioxidant capacity of mitochondria.

Compared to rotenone, metformin appears to have a dual effect characterized by a moderate inhibitor of complex 1 that affects respiration mainly during phosphorylation (see Fig. 3), and by a possible but barely detectable uncoupling effect (see Table 2). Interestingly, metformin did not increase ROS production in the presence of glutamate–malate (i.e. in the forward direction of electron flux), as it is the case with rotenone. In the presence of glutamate–malate and succinate, rotenone has a double effect on complex 1: a simultaneous increased and decreased ROS production at the forward and reverse electron pathway, respectively. On the contrary, metformin decreases the reverse flux-related ROS production but does not increase the forward flux-related ROS production.

This unique property may be related to the therapeutic properties of metformin. This drug, which possesses anti-hyperglycemic property, is currently used in the treatment of type 2 diabetes. Interestingly, a large prospective clinical survey, conducted in (Anonymous, 1998), has suggested that the beneficial effect of metformin was not only related to its action on blood glucose normalization). Indeed, if reduction of blood glucose remains as cornerstone treatment of such patients, compelling evidence shows that onset and progression of hyperglycemia-induced vascular complications is associated with mitochondria-generated oxidative stress (Brownlee, 2001; Du et al., 2000, 2003; Nishikawa et al., 2000). Because we have provided experimental data showing that complex 1 inhibition prevented oxidative-stress-related cell death (Chauvin et al., 2001), we hypothesized that metformin might also exhibit such property. Indeed, therapeutic concentration of metformin (100 μM) prevents oxidative stress- and hyperglycemia-induced cell death in several cell lines (Guigas et al., 2004), including in human endothelial cells (Detaille et al., 2005). The data presented here add new insights in our attempt to understand the intrinsic mechanism by which metformin prevent glucose toxicity besides its lowering blood glucose property.

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