Opening of the Mitochondrial Permeability Transition Pore Induces Reactive Oxygen Species Production at the Level of the Respiratory Chain Complex I

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We have investigated the consequences of permeability transition pore (PTP) opening on the rate of production of reactive oxygen species in isolated rat liver mitochondria. We found that PTP opening fully inhibited H$_2$O$_2$ production when mitochondria were energized both with complex I or II substrates. Because PTP opening led to mitochondrial pyridine nucleotide depletion, H$_2$O$_2$ production was measured again in the presence of various amounts of NADH. PTP opening-induced H$_2$O$_2$ production began when NADH concentration was higher than 50 μM and reached a maximum at over 300 μM. At such concentrations of NADH, the maximal H$_2$O$_2$ production was 4-fold higher than that observed when mitochondria were permeabilized with the channel-forming antibiotic amphotericin, indicating that the PTP opening-induced H$_2$O$_2$ production was not due to antioxidant depletion. Moreover, PTP opening decreased rotenone-sensitive NADH ubiquinone reductase activity, whereas it did not affect the NADH FeCN reductase activity. We conclude that PTP opening induces a specific conformational change of complex I that (i) dramatically increases H$_2$O$_2$ production so long as electrons are provided to complex I, and (ii) inhibits the physiological pathway of electrons inside complex I. These data allowed the identification of a novel consequence of permeability transition that may partly account for the mechanism by which PTP opening induces cell death.

Because PTP opening led to pyridine nucleotide depletion, mitochondrial swelling is due to the presence of matrix proteins that cannot diffuse through the open pore, thus creating an oncotic pressure gradient (5). The role of PTP in the commitment to cell death is supported by (i) the fact that mitochondrial swelling leads to outer membrane rupture and release of pro-apoptotic intermembrane space proteins (6–8), (ii) the demonstration that PTP opening occurs in intact cells (9–13), and (iii) the finding that different PTP inhibitors have a protective effect in several models of cell death (9, 14–22). However, the mechanisms by which PTP opening leads to the release of pro-apoptotic proteins in vitro needs to be clarified, especially whether PTP opening induces mitochondrial swelling in intact cells (or not) when mitochondria are surrounded by cytosolic proteins. Indeed, PTP opening in the absence of cytochrome c release has been reported in intact cells (7), whereas massive cytochrome c release after PTP opening has been observed in the absence of mitochondrial swelling (23).

It has been suggested that PTP opening increases ROS production in vitro (24), which may be relevant in the commitment to cell death. However, because PTP opening leads to mitochondrial uncoupling whereas mitochondrial uncoupling has been shown to decrease ROS production (25–28), this idea did not get much attention until recently, when it was reported that PTP opening increases ROS production in isolated mitochondria (29).

To characterize the mechanism by which PTP opening increases ROS production, knowing that mitochondria produce ROS at the level of complexes I and III (30–32), we have examined in this work the role of the respiratory substrates in ROS production before and after pore opening. Surprisingly, we found that PTP opening fully inhibited the H$_2$O$_2$ production of isolated mitochondria, regardless of the respiratory substrates. Because PTP opening led to pyridine nucleotide depletion, H$_2$O$_2$ production was measured again in the presence of NADH. Under this condition, PTP opening specifically increased H$_2$O$_2$ production. Moreover, PTP opening dramatically decreased rotenone-sensitive NADH ubiquinone reductase activity. We conclude that PTP opening induces a specific conformational change of complex I that (i) dramatically increases H$_2$O$_2$ production so long as electrons are provided to complex I,.

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and (ii) inhibits the physiological pathway of electrons inside complex I.

MATERIALS AND METHODS

Rat liver mitochondria were prepared according to standard differential centrifugation procedures in a medium containing 250 mM sucrose, 20 mM Tris-HCl (pH 7.4), and 1 mM EGTA-Tris.

ROS production was assessed fluorometrically either in the presence of 20 units of horseradish peroxidase (HRP) and 100 μM homovanillic acid (HVA), as described in Ref. 33 (excitation—emission, 319—420 nm), or in the presence of 5 mM H2DCFDA, as described in Ref. 34 (excitation—emission, 503—621 nm). Pyridine nucleotide oxidation-reduction status was estimated based upon endogenous fluorescence of NAD(P)H (excitation 340 nm, emission 319 nm). Pyridine nucleotide oxidation-reduction changes at 520 nm. Fluorometric assays and light scattering measurements were performed at 30 °C simultaneously with a double-beam PTF Quamatameter C61 fluorometer.

Because the excitation and emission spectra of NADH and HVA are close, the measured signals (namely, F319—420 and F340—450) in the presence of these two fluorochromes correspond to

\[ F_{319-420} = F_{\text{HVA}}^{319-420} + F_{\text{NADH}}^{319-420} \] (Eq. 1)

\[ F_{340-450} = F_{\text{NADH}}^{340-450} + F_{\text{HVA}}^{340-450} \] (Eq. 2)

where \( F_{\text{NADH}} \) and \( F_{\text{HVA}} \) correspond to the fluorescence of NADH and HVA, respectively.

Given that \( \alpha = \frac{F_{\text{NADH}}^{319-420}}{F_{\text{HVA}}^{319-420}} \) and \( \beta = \frac{F_{\text{HVA}}^{340-450}}{F_{\text{NADH}}^{340-450}} \), then

\[ F_{\text{HVA}}^{319-420} = F_{319-420} - \alpha F_{\text{NADH}}^{319-420} \] (Eq. 3)

\[ F_{\text{NADH}}^{340-450} = F_{340-450} - \beta F_{\text{HVA}}^{340-450} \] (Eq. 4)

After the addition of NADH, which led to a sudden increase in fluorescence (ΔF) in the two channels, \( \alpha \) was determined as \( \Delta F_{\text{NADH}}^{319-420} \), \( \Delta F_{\text{NADH}}^{340-450} \) and \( \Delta F_{\text{HVA}}^{340-450} \). The experimental value of \( \alpha \) was 0.161 ± 0.013 (mean ± S.E., \( n = 63 \)). Importantly, \( \alpha \) remained constant in the range of concentrations used (see Fig. 2). Under our experimental conditions, H2O2 addition led to a sudden increase in fluorescence in \( F_{319-420} \) but not in \( F_{340-450} \) (data not shown), indicating that \( \beta \) was almost equal to zero. Therefore, H2O2 production (i.e. \( F_{\text{HVA}}^{319-420} \)) was obtained after the deconvolution of the two channels using the following equation

\[ F_{\text{HVA}}^{319-420} = F_{319-420} - \alpha F_{\text{NADH}}^{319-420} \] (Eq. 5)

Mitochondrial oxygen consumption was measured polarographically at 30 °C using a Clark-type oxygen electrode. Complex I activity was assessed either by measuring the oxidation rate of NADH in the presence of an artificial electron acceptor, DuB (NADH DuB reductase activity), or by measuring the reduction rate of FeCN (NADH FeCN reductase activity) as described in Ref. 36. The absorbance changes of NADH and FeCN were measured at 340 and 410 nm, respectively, with a Uvikon-Kontron 941 plus spectrophotometer equipped with magnetic stirring and thermostatic control.

Succinic acid, rotenone, valinomycin, alamethicin, HRP, HVA, CCCP, DuB, FeCN, EGTA, Tris, HCl, glutamic acid, CaCl2, and CsA were purchased from Sigma-Aldrich; phosphoric acid, sucrose, malic acid, and NADH were purchased from Merck. KCN was purchased from ProLabo, and H2DCFDA was obtained from Molecular Probes.

Results are expressed as mean ± S.E. Statistically significant differences were assessed by a Student’s t test (Stat View, Abacus Concepts, Inc., Berkeley, CA).

RESULTS

Effect of PTP Opening on ROS Production—In the experiment in Fig. 1, we measured the production of H2O2 before and after PTP opening, with mitochondria energized either with glutamate plus malate (gray traces) or succinate (in the absence of rotenone) (black traces). Because the HRP/HVA method is known to be sensitive to NADH auto-fluorescence,
Fig. 2. Relationship between NADH concentration and NADH fluorescence. Experimental conditions were as in Fig. 1. Experiments were started by the addition of 1 mg mitochondria followed by the addition of 400 μM Ca^{2+} to open the PTP (not shown). A, ΔF_{340–450} (C) and ΔF_{319–420} (D) represent the fluorescence in the presence of the indicated concentration of NADH minus the fluorescence before NADH addition. Results represent one typical experiment. B, relationship between ΔF_{NADH}/ΔF_{NADH} and NADH concentration. Results are mean ± S.E. (n = 9).

We simultaneously recorded NADH (340–450 nm) and the HVA (319–420 nm) fluorescence. In agreement with previous reports (28, 37, 38), the production of H_{2}O_{2} before PTP opening was higher in the presence of complex II than complex I substrates (Fig. 1, lower signal), whereas the NADH signal remained stable (Fig. 1, upper signal). As expected, PTP opening led to NADH oxidation, which interfered with the HVA signal. However, once the NADH signal had reached a new steady state (i.e. when NADH did not affect the slope of the HVA signal), the production of H_{2}O_{2} was almost undetectable. Therefore, PTP opening decreased the H_{2}O_{2} production of isolated mitochondria regardless of the respiratory substrates.

Because pyridine nucleotides diffuse out of mitochondria after PTP opening, which leads to the inhibition of mitochondrial respiration, we next determined whether the decrease in H_{2}O_{2} production was related to a lack of NADH by measuring H_{2}O_{2} production after the addition of different amounts of NADH. In this particular condition, keeping in mind that NADH is consumed after PTP opening, whereas NADH fluorescence interferes with the HVA signal, it must be noted that fluorescence quenching occurred in both channels (i.e. 340–450 and 319–420) when NADH concentration increased (Fig. 2A). Thus, the consumption of NADH induced by PTP opening led to a decrease in NADH fluorescence below 200 μM but to an increase above 200 μM (data not shown). Therefore, F_{319–420} underestimated and overestimated the apparent production of H_{2}O_{2} when NADH consumption occurred below and above 200 μM, respectively. On the other hand, ΔF_{340–450}/ΔF_{NADH} remained constant whatever the concentration of NADH used (Fig. 2B). Consequently, the production of H_{2}O_{2} could be precisely determined by deconvolution of the two channels, as indicated under “Materials and Methods.”

As shown in Fig. 3A, in the presence of 150 μM NADH, PTP opening led to NADH consumption (trace a) and to an increase in the HVA signal (trace b). As expected, the addition of NADH did not interfere with H_{2}O_{2} production after deconvolution of the two channels (trace c), and the production of H_{2}O_{2} induced by PTP opening was then easily observed. Similar experiments were performed with various concentrations of NADH. As shown in Fig. 3B, PT-induced H_{2}O_{2} production (•) started when NADH concentration was higher than 50 μM and was maximal from 300 μM.

To determine whether or not PT-induced H_{2}O_{2} production was related to a leak of antioxidant through the open pore, we measured the production of H_{2}O_{2} after the inner membrane had been permeabilized with the channel-forming antibiotic alamethicin. Although alamethicin induces large pores in the inner membrane, which leads to mitochondrial swelling and NADH oxidation, the maximal H_{2}O_{2} production induced by alamethicin (Fig. 3B, open squares) represented approximately one-quarter of that after PTP opening. Therefore, a large part of the PT-induced H_{2}O_{2} production was specifically related to PTP opening.

Using the H_{2}O_{2}-sensitive probe H_{2}DCFDA, it has been reported previously that PTP opening induced ROS production in the absence of NADH addition (29). Because this observation was in complete disagreement with the results in Fig. 1, we next measured the effect of PTP opening on the rate of H_{2}DCFDA oxidation.

Because mitochondrial volume changes optimally affect light scattering at 520 nm, which is close to the excitation-emission wavelengths recommended for H_{2}DCFDA, we first checked whether mitochondrial swelling could affect the signal assessed with this probe. In the experiment depicted in Fig. 4, we simultaneously recorded H_{2}DCFDA oxidation (upper signal, 503–521 nm) and light scattering (lower signal, 520–520 nm) when mitochondrial volume was modified by K^{+} movements across the inner membrane, a condition where mitochondrial swelling is not due to PTP opening. As expected, the addition of K^{+} ionophore valinomycin led to mitochondrial swelling as a consequence of the ΔΨ-driven accumulation of K^{+} inside mitochondria, whereas the addition of uncoupler CCCP led to mitochondrial shrinkage as a result of ΔΨ release after ΔΨ had been abolished. As shown in Fig. 4, such changes of mitochondrial volume interfered with the signal coming from the H_{2}DCFDA probe (503–521 nm). However, because the slope of the signal remained similar to the control when mitochondrial volume had stabilized, H_{2}DCFDA oxidation seemed to be a convenient assay of ROS production, except during volume transition.

In the experiments in Fig. 5, we measured H_{2}DCFDA oxidation before and after PTP opening, with mitochondria energized either with glutamate plus malate (gray traces) or succinate (in the absence of rotenone) (black traces). As can be seen, the rates of H_{2}DCFDA oxidation were the same before PTP opening regardless of the respiratory substrates. Because in non-phosphorylating mitochondria, the production of ROS is expected to be higher when mitochondria are energized with complex II rather than complex I substrates (28, 37, 38) (see Fig. 1), this finding strongly suggested that an auto-oxidation of H_{2}DCFDA occurred in our conditions of incubation. Nevertheless, as shown in Fig. 5, PTP opening led to a dramatic but transient increase in H_{2}DCFDA oxidation when mitochondria were energized with NAD-linked substrates (gray trace). On the other hand, PTP opening did not affect H_{2}DCFDA oxidation when electrons were provided directly to complex II (black trace).

Effect of PTP Opening on Complex I—Because mitochondria produce ROS at the level of complexes I and III, we next studied the effect of PTP opening on the respiratory chain. In the experiment in Fig. 6, we measured the oxygen consumption rate of liver mitochondria incubated in the presence of glu-
mate plus malate (panel A) or succinate (panel B). Because PTP opening as well as alamethicin addition led to the release of pyridine nucleotides, which sets a limit for respiration (Fig. 6, Insert), the experiments were performed in the presence of 1 mM NADH when the NAD-linked substrates were used (Fig. 6A). As expected, both PTP opening and alamethicin addition led to a sustained increase in respiration. It must be noted however, that alamethicin-induced respiration was 2-fold that induced by PTP opening in the presence of complex I substrates (panel A). On the other hand, alamethicin- and PT-induced respiration were the same in the presence of succinate (panel B), which demonstrated that PTP opening did not affect the respiratory chain activity downstream from complex II. Importantly, the addition of alamethicin after PTP opening when

FIG. 3. Effect of NADH on H₂O₂ production rate after PTP opening. Experimental conditions were as in Fig. 1. The final volume was 2 ml, pH 7.4, 30 °C. A, experiments were started by the addition of 1 mg of mitochondria (data not shown). Where indicated, 150 mM NADH and 400 μM Ca²⁺ were added. Deconvolution was performed as indicated under "Materials and Methods." B, H₂O₂ production in the presence of the indicated concentrations of NADH either after PTP opening (●) or after addition of 1.5 μM alamethicin (□). Results are mean ± S.E. (n = 6 for PTP opening, n = 3 for addition of alamethicin).

FIG. 4. Effect of mitochondrial volume on H₂DCFDA oxidation. The incubation medium contained 250 mM sucrose, 100 μM EGTA, 20 mM Tris-HCl, 5 mM glutamate-Tris, 2.5 mM malate-Tris, and 5 mM H₂DCFDA. The final volume was 2 ml, pH 7.4, 30 °C. Experiments were started by the addition of 2 mg of mitochondria (data not shown). Where indicated, 5 mM potassium P_i, 1.25 μg/ml valinomycin, and 250 nM CCCP were added. The dashed line denotes H₂DCFDA oxidation in the absence of valinomycin and CCCP.

FIG. 5. Effect of PTP opening on H₂DCFDA oxidation. The incubation medium contained 250 mM sucrose, 10 μM EGTA, 20 mM Tris-HCl, 5 μM H₂DCFDA, and either 5 mM glutamate-Tris plus 2.5 mM malate-Tris (gray traces) or 5 mM succinate-Tris (black traces). The final volume was 2 ml, pH 7.4, 30 °C. Experiments were started by the addition of 2 mg of mitochondria (data not shown). Where indicated, 5 mM P_i-Tris and 150 μM Ca²⁺ were added.
electrons were provided to complex I (panel A) did not further increase the respiratory rate, excluding the presence of a sub-population of mitochondria in which PT would not have occurred and suggesting that either NADH diffusion across the inner membrane or NADH oxidation at the level of complex I was kinetically controlled under this particular condition.

We next directly measured complex I activity using two different artificial electron acceptors (namely, FeCN or DUb) in the presence of complex IV inhibitor KCN. Mitochondrial inner membrane was made permeable to NADH either by PTP opening or by osmotic shock that led to inner membrane rupture. As shown in Fig. 7A, FeCN reduction rates were similar regardless of the way NADH entered mitochondria, indicating that NADH diffusion through the open pore did not control NADH oxidation rate. However, when electrons were transferred from NADH to a ubiquinone analogue DUb, total NADH oxidation rate (Fig. 7B) decreased after pore opening but rotenone-insensitive NADH oxidation rate (Fig. 7C) did not. Therefore, PTP opening dramatically inhibited rotenone-sensitive NADH ubiquinone reductase activity.

Finally, because complex I inhibition is known to increase ROS production in the presence of complex I substrates, we compared the effect of PTP opening and rotenone addition on the production of H$_2$O$_2$. As shown in Fig. 8, PT-induced H$_2$O$_2$ production in the presence of 500 μM NADH (black trace) was much higher than the production of H$_2$O$_2$ in the presence of rotenone and NAD-linked substrates (gray trace).

**DISCUSSION**

In this work, we have shown that PTP opening directly affected electron-transfer through complex I and induced ROS production in the presence of NADH.

Respiratory Inhibition after PTP Opening—It has been reported that PT leads to a decrease in oxygen consumption rate when mitochondria are energized with NAD-linked substrates, a finding previously imputed to pyridine nucleotide depletion through the open pore (39). In this work, we show that complex I activity remains partly inhibited after PTP opening, even in the presence of saturating amounts of NADH (Fig. 6, Inset). It must be noted that such inhibition is not due to a kinetic control during NADH diffusion across the inner membrane, because under this condition (i.e. after PTP opening and in the
The incubation medium contained 250 mM sucrose, 10 mM EGTA, 20 mM Tris-HCl, 20 units HRP, 100 μM HVA, 5 mM P1-Tris, and 5 mM glutamate-Tris plus 2.5 mM malate-Tris and then challenged for 10 min with 100 μM Ca2+. PT opening was checked by mitochondrial swelling. For hypoosmotic shock-induced inner membrane rupture, 50 μg (A) or 250 μg (B and C) of mitochondria were pre-incubated in the medium of Fig. 6 supplemented with 5 mM glutamate-Tris plus 2.5 mM malate-Tris and then challenged for 10 min with 100 μM Ca2+. PT opening was checked by mitochondrial swelling. For PTP opening, 50 μg (A) or 250 μg (B and C) of mitochondria were pre-incubated for 10 min in a hypoosmotic medium containing only 5 mM glutamate-Tris plus 2.5 mM malate-Tris, pH 7.4. The medium was supplemented with stock solutions to be identical to the medium in which the PTP opening was performed. The final volume was 2 ml, pH 7.4, 30 °C. The incubation medium was then supplemented with 1.5 mM KCN (all panels) and 2 μM rotenone (C). FeCN reduction (A) and NADH oxidation (B and C) were measured after the addition of 500 μM FeCN plus 200 μM NADH (A) or 200 μM NADH plus 150 μM DUb. Results are means ± S.E. of at least seven separate determinations. **, p < 0.01, Student’s t test.

ROS Production Induced by PTP Opening

Mechanism of ROS Production after PT—The two methods used in this work to assess the mitochondrial production of H2O2 (namely, HRP/HVA and H2DCFDA) gave noticeably different results. H2DCFDA is widely used for measuring H2O2 in intact cells, and indeed, PT-induced ROS production was described using this probe (24, 29). It must be kept in mind, however, that H2DCFDA is prone to auto-oxidation. On the contrary, the HRP/HVA technique cannot be used in intact cells but is a reference method for H2O2 production in isolated mitochondria.

With this latter technique, PT opening inhibited H2O2 production (Fig. 1) unless NADH was added (Fig. 3), regardless of the respiratory substrate used before PT. These findings are in total agreement with numerous works showing that (i) ROS electrons can be transferred to electron acceptors that bind complex I at non-physiologic sites. Such electron flux is not coupled to proton pumping and is rotenone insensitive; thus, it does not represent the physiologic electron pathway inside complex I (40, 41). Among artificial electron acceptors, DUb binds simultaneously physiologic and non-physiologic sites, whereas FeCN binds non-physiologic site(s) only (36). The fact that PTP opening decreased the rotenone-sensitive NADH DUb reductase activity indicates that PTP opening per se inhibits the physiologic pathway of electrons inside complex I. Although the diameter of the open PTP is comparable with that of the pores induced by alamethicin (5, 42), the fact that alamethicin addition did not lead to complex I inhibition in the presence of NADH indicates that the PT-induced complex I inhibition is not due to the release of a putative complex I activating factor. Therefore, the PT-induced complex I inhibition is most probably due to a conformational change of complex I. It should be noted that PT did not affect either the NADH FeCN or the rotenone-insensitive NADH DUb reductase activities, which suggests that such conformational change does not affect the affinity of complex I for NADH.

Complex I catalyzes the transfer of electrons from NADH to the ubiquinone pool with an electron flux that is coupled to proton-pumping and inhibited by rotenone (40, 41). However, at non-physiologic sites. Such electron flux is not coupled to proton pumping and is rotenone insensitive; thus, it does not represent the physiologic electron pathway inside complex I (40, 41). Among artificial electron acceptors, DUb binds simultaneously physiologic and non-physiologic sites, whereas FeCN binds non-physiologic site(s) only (36). The fact that PTP opening decreased the rotenone-sensitive NADH DUb reductase activity indicates that PTP opening per se inhibits the physiologic pathway of electrons inside complex I. Although the diameter of the open PTP is comparable with that of the pores induced by alamethicin (5, 42), the fact that alamethicin addition did not lead to complex I inhibition in the presence of NADH indicates that the PT-induced complex I inhibition is not due to the release of a putative complex I activating factor. Therefore, the PT-induced complex I inhibition is most probably due to a conformational change of complex I. It should be noted that PT did not affect either the NADH FeCN or the rotenone-insensitive NADH DUb reductase activities, which suggests that such conformational change does not affect the affinity of complex I for NADH.

Mechanism of ROS Production after PT—The two methods used in this work to assess the mitochondrial production of H2O2 (namely, HRP/HVA and H2DCFDA) gave noticeably different results. H2DCFDA is widely used for measuring H2O2 in intact cells, and indeed, PT-induced ROS production was described using this probe (24, 29). It must be kept in mind, however, that H2DCFDA is prone to auto-oxidation. On the contrary, the HRP/HVA technique cannot be used in intact cells but is a reference method for H2O2 production in isolated mitochondria.

With this latter technique, PT opening inhibited H2O2 production (Fig. 1) unless NADH was added (Fig. 3), regardless of the respiratory substrate used before PT. These findings are in total agreement with numerous works showing that (i) ROS
production decreases with the mitochondrial membrane potential when electrons are provided to complex II (25–28), or (ii) electrons are not provided anymore to complex I because of NADH leak through the open pore (39, 43). On the contrary, PTP opening transiently increased or did not affect H$_2$DCFDA oxidation in the presence of complex I and complex II substrates, respectively (Fig. 5). The fact that PTP opening totally inhibited H$_2$O$_2$ production in the absence of NADH, whereas it did not decrease H$_2$DCFDA oxidation in the same condition, is most probably because of a large auto-oxidation of that probe. The reason why PTP opening transiently increased H$_2$DCFDA oxidation in the presence of glutamate plus malate remains unclear. However, because H$_2$DCFDA oxidation was not affected by catalase addition (data not shown), we propose that PTP opening may increase H$_2$DCFDA auto-oxidation under this particular condition of incubation, independently of any production of ROS by the respiratory chain.

Because the previously published works reporting that PTP opening induces ROS production have been performed with H$_2$DCFDA (24), the question arises as to whether PTP opening really induces H$_2$O$_2$ production in intact cells. It must, however, be kept in mind that the mean concentration of NADH in cells is within the millimolar range, a concentration at which PT-induced H$_2$O$_2$ production occurs (Fig. 3).

Mitochondria produce ROS at the level of complexes I and II (30–32). The fact that PT did not induce ROS production when electrons were provided directly to complex II (i.e. downstream complex I) strongly suggests that the PT-induced H$_2$O$_2$ production observed in the presence of NADH occurred in complex I but not in complex III.

Because complex I inhibitors have been shown to increase ROS production in the presence of NAD-linked substrates (26, 28, 44, 45), it was tempting to speculate that the PT-induced ROS production observed in the presence of NADH was due to the PT-induced inhibition of complex I. However, as shown in Fig. 8, rotenone slightly favored H$_2$O$_2$ production, whereas PTP opening dramatically increased H$_2$O$_2$ production. Because complex I inhibition could not per se account for the PT-induced ROS production, we propose that PTP opening induces a specific conformational change of complex I that dramatically increases H$_2$O$_2$ production so long as electrons are provided to complex I, and inhibits the physiologic pathway of electrons inside complex I. Moreover, depending on the incubation conditions, it may also catalyze H$_2$DCFDA oxidation in the absence of NADH.

**Implications for PTP Opening-induced Cell Death**—It has long been known that oxidative stress can induce cell death (46). Although ROS can trigger PTP opening both in *vitro* (5) and in intact cells (9), ROS have been shown to induce cytochrome c release and subsequent cell death, whereas PT remained closed (47). Evidence suggests that in the absence of outer membrane rupture, the release of pro-apoptotic factors occurs by means of large pores that are the result of the insertion of Bcl-2 family protein BAX into the outer membrane (1–3). Voltage-dependent anion channel (VDAC) has also been implicated, alone or in interaction with Bcl-2 family proteins, in outer membrane permeabilization (48–50). In support of the idea that ROS affect outer membrane permeability, it has been reported that (i) H$_2$O$_2$ induced BAX translocation in cardiac myocytes (51), (ii) NO$^-$-induced BAX translocation in SH-SY5Y neuroblasts (52), and (iii) -O$_2$ induced VCAD-dependent cytochrome c release in HepG2 cells (48). Moreover, it has been shown that ROS production induced by the addition of transforming growth factor β decreased the expression and level of anti-apoptotic protein Bcl-xL in fetal hepatocytes (53).

Although cell death does not necessarily require PTP open-

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