Rotenone Enhances the Antifungal Properties of Staurosporine

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We studied staurosporine-induced cell death in the filamentous fungus Neurospora crassa. The generation of reactive oxygen species during the process appears to be an important signaling event, since addition of the antioxidant glutathione prevents the effects of staurosporine on fungal growth. Selected mutants with mutations in respiratory chain complex I are extremely sensitive to the drug, stressing the involvement of complex I in programmed cell death. Following this finding, we determined that the complex I-specific inhibitor rotenone used in combination with staurosporine results in a synergistic and specific antifungal activity, likely through a concerted action on intracellular glutathione depletion. Paradoxically, the synergistic antifungal activity of rotenone and staurosporine is observed in N. crassa complex I mutants and in Saccharomyces cerevisiae, which lacks complex I. In addition, it is not observed when other complex I inhibitors are used instead of rotenone. These results indicate that the rotenone effect is independent of complex I inhibition. The combination of rotenone and staurosporine is effective against N. crassa as well as against the common pathogens Aspergillus fumigatus and Candida albicans, pointing to its usefulness as an antifungal agent.

Programmed cell death (PCD) refers to a genetically controlled process of cellular suicide initiated by endogenous or extrinsic signals. Many of the genes involved are widely conserved from unicellular to multicellular organisms (46). Apoptosis and autophagy, with its particular characteristics, have been recognized as the main categories of PCD (27). The process of PCD is crucial for the development and homeostasis of metazoan organisms and has been implicated in a number of human disorders, including cancer and neurodegenerative and infectious diseases (3, 10, 25, 55).

The participation in PCD of mitochondria, the cellular organelles responsible for the production of most cellular ATP in eukaryotes (30), has been well established. Particularly, these organelles have a central role in the intrinsic (mitochondrion-dependent) pathway of apoptosis, which includes production of reactive oxygen species (ROS), membrane depolarization, ultrastructural changes, and the release of cytochrome c and other proteins (18, 50, 55). Drugs like staurosporine (STS), an inhibitor of protein kinases, have been used to induce the mitochondrial-dependent pathway of apoptosis (24, 35). Staurosporine (48) and derivatives have been used in clinical trials for cancer therapy (63). The complex I inhibitor rotenone too has been widely used to induce PCD and also extensively applied as a pesticide (11, 39, 56). Thus, these types of drugs can be employed for the acquisition of fundamental knowledge and for more practical applications, like modulation of the progression of PCD.

Modulation of PCD by targeting metabolic pathways involved in the process can be exploited to the benefit of human health in several very significant situations, from cancer therapy (4, 57) to the treatment of fungal infections (3, 52). However, the molecular basis of PCD involves complex metabolic networks (32, 38, 59), and further work is required for their identification. Neurospora crassa has many advantages for biochemical and genetic experiments (14, 17, 23) and is thus a good model organism for the study of mechanisms of PCD. We are interested in identifying the cell molecular pathways associated with PCD and using this knowledge to devise strategies to modulate the process. In this work, we analyze the effects of STS on the N. crassa wild type and mitochondrial complex I mutants and describe the synergistic effect of combining STS and rotenone on this organism and other human-pathogenic fungi.

MATERIALS AND METHODS

Strains and growth techniques. The wild-type N. crassa sequenced strain (FGSC 2489) and several deletion strains generated by the Neurospora Genome Project (17) were obtained from the Fungal Genetics Stock Center (43). Mutants with mutations in the respiratory chain complex I genes nuo14, nuo51, and nuo78 have been described previously (41). Standard procedures were employed for growth and handling of the Neurospora strains (13). Aspergillus fumigatus ATCC 46645 was a kind gift of Eugénia Pinto. Spore suspensions were prepared from 5-day-old cultures grown on Sabouraud’s glucose agar at 37°C. The cells were harvested by gentle agitation with 0.01% (vol/vol) Tween 80 and filtered through cheesecloth. Candida albicans SC5314 was a kind gift of Alexandra Correia. The cells were incubated in yeast Winge liquid medium at 30°C, agitated at 200 rpm overnight, harvested by centrifugation, and resuspended in saline buffer (51). Saccharomyces cerevisiae was a kind gift of Vitor Costa. The cells were incubated in yeast extract-peptone-dextrose (YPD) liquid medium and agitated at 140 rpm overnight at 26°C.

Cell viability. Neurospora wild-type conidial cells were harvested and suspended in minimal medium at a concentration of 5 × 10⁵ cells/ml and incubated for 30 min at 26°C. Then, cells were washed in water, and staurosporine (STS) from LC Laboratories was added from a stock solution of 5 mg/ml in dimethyl sulfoxide (DMSO) (DS879: Sigma-Aldrich). Cells incubated with DMSO were
used as a control. The viabilities of conidia at different times were determined by plating aliquots of the cells on GFS agar medium (13), which induces colonial growth, and counting CFU after incubation of the plates at 26°C for about 72 h.

**Detection of ROS.** Mitochondrial ROS production was monitored by using dihydrohodamine 123 from Sigma (St. Louis, MO). The reduced form of dihydrohodamine 123 does not fluoresce until entering an actively respiring cell, where it is oxidized by ROS (mainly H2O2, but also HOCl and peroxinitrite) to a green fluorescent compound that is sequestered in the mitochondria (36). N. crassa conidial cells were harvested and suspended (5 × 10⁶ cells/ml) in minimal medium containing 50 μg/ml of dihydrohodamine 123. The cell suspensions were incubated at 26°C for 30 min and washed with water before the addition of STS. Rhodamine 123 fluorescence was monitored at the single-cell level by use of a FACSCalibur cytometer (BD Biosciences), and data were analyzed with CELLQuest version 3.3 (BD Biosciences), with excitation at 480 nm and emission at 530 nm. When employed, antioxidants were added in preincubation with dihydrohodamine 123 and (after washes) added again in conjunction with STS.

**Glutathione determination.** N. crassa (10⁷ conidia/ml) was grown in minimal medium for 5 h at 30°C, followed by 60 min of incubation in the presence of 12.5 μM staurosporine, 100 μg/ml rotenone, both, or DMSO as a control. The cells were collected by filtration and disrupted by maceration. Then, EDTA was added to a final concentration of 5 mM and the material was suspended in 1 M HClO₄, 50 mM potassium phosphate buffer, pH 7.2, and centrifuged for 5 min at 5,000 × g at 4°C. The supernatant was neutralized to pH 6 to 7 with 4 M KOH and 0.6 M MOPS (morpholinopropanesulfonic acid) and centrifuged for 1 min in maximum rotation at 4°C. The sum of glutathione and glutathione disulfide (total glutathione) was determined using a kinetic assay with glutathione reductase, described before (1). The reaction was followed spectrophotometrically using a Shimadzu UV-160A instrument.

**TUNEL assay.** N. crassa conidia were preincubated in minimal medium for 30 min, washed in water, and then treated with staurosporine for 60 min and washed again. Spheroplasts were prepared, and fixation was performed 5 h after treatment with the drug. DNA strand breaks were analyzed by terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) using an in situ cell death detection fluorescein kit (Roche Applied Science), essentially as described previously (8). Flow cytometry, using 20,000 cells per sample, was carried out as described above.

**Spot assays.** Cellular suspensions (conidia) from all fungi were prepared and adjusted to a concentration of 6.56 × 10⁶ cells/ml. Five microliters from serial dilutions of each fungal cell preparation was spotted on agar plates containing various drugs. Images were taken by scanning the plates after 2 to 3 days of incubation with the drug. DNA strand breaks were analyzed by terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) using an in situ cell death detection fluorescein kit (Roche Applied Science), essentially as described previously (8). Flow cytometry, using 20,000 cells per sample, was carried out as described above.

**RESULTS**

**Selected complex I mutants are highly sensitive to staurosporine.** Staurosporine is a cell death inducer for a variety of cells. To assess whether it has antifungal activity against *Neurospora*, we studied the effects of various concentrations of STS (5 to 50 μM) on the viability of asexual spores (conidia). Conidial cells were exposed to STS for different time periods and plated in GFS agar medium (which induces colonial growth of *N. crassa*), and the resulting colonies were counted. As shown in Fig. 1A, the viability of cells is not significantly affected by DMSO (control) or a low STS concentration (5 μM). In contrast, cell viability decreases drastically with higher concentrations of STS, in a concentration-dependent manner, showing that STS has potent antifungal activity against *N. crassa* conidia. As detected by the TUNEL assay (Fig. 1B), STS treatment also provokes chromatin fragmentation, a hallmark of apoptosis, indicating that it induces an apoptosis-like cell death in *N. crassa*.

We found previously that mutants with mutations in respiratory chain complex I are more resistant to another cell death inducer (phytosphingosine) than the wild-type strain (8). We therefore asked whether complex I is also involved in STS-induced death. We analyzed the STS sensitivities of conidial cells from complex I mutant strains in spot assays. These mutants display different phenotypes in terms of complex I assembly (41, 58). Serial dilutions of conidial suspensions from each strain were spotted on media containing STS and incubated at 26°C, and the growth levels of the strains were qualitatively analyzed (Fig. 2). All strains grew similarly on control GFS agar medium but showed differences when STS was included in the medium. The wild-type and *nuo78* mutant strains were affected by STS to similar extents. In contrast, *nuo9.8, nuo14, nuo30.4*, and *nuo51* complex I mutants were extremely sensitive to the drug. These results show that complex I (and mitochondria) is involved in PCD induced by both phytosphingosine and staurosporine but that the two drugs act by quite different mechanisms, since all of these mutants were more resistant to phytosphingosine than the wild-type strain (8).

**Rotenone and STS display a specific and synergistic activity against Neurospora and other pathogenic fungi.** Since we observed that selected complex I mutants are highly sensitive to...
STS (Fig. 2), we reasoned that inhibiting the respiratory chain enzyme in wild-type cells would also render them much more sensitive to STS-induced death. Indeed, Fig. 2 shows that staurosporine has a much stronger antifungal effect against wild-type *N. crassa* cells when the complex I inhibitor rotenone is incorporated in the medium. While rotenone alone has no detectable effect, its use in combination with STS leads to a dramatic synergistic effect on fungal growth.

To determine whether the synergistic interaction of rotenone and STS was specific for *Neurospora* or also affected growth of other fungi, we tested the effects of these drugs on two common human pathogens, *A. fumigatus* and *C. albicans*. Figure 3 depicts the results obtained from spot assay experiments. Treatment with STS alone reduced viability in all three species, although rotenone alone had little effect on viability of these fungal species. In contrast, rotenone and STS highly reduced the viability of all three fungi. Taken together, these results point out that this type of drug combination may be very useful as a potential antifungal agent.

In similar experiments, other combinations of death inducers and complex I inhibitors were also tested. As death inducers, we used amphotericin B, a polyene antibiotic often used for treatment of systemic candidiasis or aspergillosis (52); phytosphingosine, with established antifungal activity against *N. crassa* and *Aspergillus nidulans* (8, 9); and the oxidative stress agent hydrogen peroxide (44). As complex I inhibitors, we employed diphenyleneiodonium (DPI), a rather nonspecific inhibitor of NADH dehydrogenases (2, 37), and piericidin A, which interferes with the ubiquinone binding site of the enzyme, as does rotenone (20, 47). We used concentrations of death inhibitors that led to a visible effect on fungal growth. The results are summarized in Table 1. We did not observe any

### TABLE 1. Antifungal effects of drug combinations, as determined by spot assays

| Organism and complex I inhibitor | Effect achieved with death inducer: |
|----------------------------------|------------------------------------|
|                                  | STS | PHS | AMB | H$_2$O$_2$ |
| **Neurospora**                   |     |     |     |           |
| Rotenone                         | +   |     |     |           |
| DPI                              | –   |     |     |           |
| Piericidin A                     | –   | ND$^b$ | ND | ND |
| **Aspergillus or Candida**       |     |     |     |           |
| Rotenone                         | +   |     |     |           |

$^a$ Different combinations of complex I inhibitors and cell death inducers were tested. A plus sign indicates that a synergistic effect was observed, and a minus sign means that the drug combination did not display an antifungal activity higher than that achieved by each drug alone. Several concentrations of most drugs were tested, in the following ranges: amphotericin B (AMB), 0.1 to 32 µg/ml; diphenyleneiodonium (DPI), 5 to 100 µM; piericidin A, 5 to 10 µM; H$_2$O$_2$, 0.5 to 1.5 mM; phytosphingosine (PHS), 5 to 12.5 µg/ml; rotenone, 100 to 300 µg/ml. The STS concentration was 5 µM.

$^b$ ND, not determined.
synergistic antifungal effect with any of these combinations of complex I inhibitors and death inducers. These data suggested that the specific synergistic antifungal effect of rotenone and staurosporine is not dependent on the inhibition of complex I (also see below).

The antioxidant glutathione prevents the effects of STS and rotenone. The generation of ROS is a typical event occurring during programmed cell death (55). In addition, we recently identified an association between decreased ROS production in N. crassa complex I mutants and the increased resistance of these mutants to cell death induced by phyto sphingosine (8). Therefore, we decided to examine by flow cytometry the production of ROS during STS-induced cell death. We also wanted to determine whether the differential susceptibilities of the complex I mutants were correlated with the production of variable amounts of ROS among these strains. Figure 4A reveals that N. crassa conidia produce ROS upon exposure to STS. A time course analysis shows that the kinetics of ROS production is both concentration and time dependent (Fig. 4B). ROS were already detectable at 15 min, and a maximum of ~50% cells producing ROS was reached around 60 min after the drug treatment.

Next, we analyzed ROS production of complex I mutants following treatment with STS, in a similar time course experiment. The results shown in Fig. 4C revealed that all complex I mutants produce less ROS than the wild-type strain following STS treatment, similarly to what was observed when these strains were treated with the death inducer phyto sphingosine (8). These results are in agreement with findings that complex I represents a major cellular source of ROS within the cell (26). The mutants display different phenotypes in terms of complex I assembly. The nuo51 mutant assembles a nonfunctional enzyme (19), the nuo78 mutant assembles only the membrane arm (29), and the nuo14 mutant assembles the peripheral arm and intermediates of the membrane arm of complex I (41). Thus, their sensitivity toward STS (Fig. 2) cannot be correlated with the levels of ROS production following drug exposure (Fig. 4C) or with their complex I phenotypes.

In order to verify the significance of STS-induced ROS formation, we performed spot assay experiments that included several antioxidants in the culture medium. Figure 5A shows that addition of the antioxidant glutathione (or its precursor N-acetylcysteine [not shown]) reverted not only the antifungal activity of STS but also the much stronger antifungal activity of STS plus rotenone in N. crassa cells. Other antioxidants tested were ineffective, but unlike glutathione and N-acetylcysteine, they were also unable to prevent STS-induced ROS formation (Fig. 5B). These experiments suggest that ROS formation is an important event for STS-induced cell death.

The rotenone effect is independent of complex I inhibition. The idea of combining rotenone and STS came from the findings that some complex I mutants were very sensitive to STS (Fig. 2). The fact that the nuo78 mutant, which lacks the 78-kDa protein, is as sensitive to STS as the wild-type strain could be explained. Cleavage of the 78-kDa homologue was claimed to be required for apoptosis in mammalian cells (54). Thus, we assumed that inhibition of complex I by rotenone was responsible for the synergistic antifungal effect of STS plus rotenone. This reasoning implies that STS plus rotenone would have no synergy when applied to complex I mutants, because complex I is already nonfunctional in these strains. However, replication of the previous experiments confirmed that combining STS and rotenone leads to a synergistic antifungal effect on complex I mutants (Fig. 5A). Application of glutathione reverted this effect and also prevented the extreme sensitivity of the nuo51 mutant to STS. Taken together with the observations that rotenone enhances STS-induced death but that other complex I inhibitors do not (Table 1), these results indicate that another property of rotenone is actually responsible for the synergistic antifungal effect of STS plus rotenone and that this effect is independent of complex I inhibition.

To test this hypothesis, we evaluated the sensitivities to STS, rotenone, and STS plus rotenone in a species that lacks complex I, the yeast S. cerevisiae (49). As shown in Fig. 6, S. cerevisiae shows little sensitivity to either STS or rotenone alone. However, when S. cerevisiae cells were treated with a combination of STS and rotenone, viability was greatly reduced. As with N. crassa, the addition of glutathione prevented this antifungal effect and cell viability was restored to wild-type levels. These results support the findings that the synergistic antifungal effect of STS plus rotenone is independent of complex I inhibition.

Unlike STS, rotenone does not induce ROS production in N. crassa (Fig. 7A). The specific protection by glutathione from the STS and rotenone effects led us to determine the intracellular levels of the antioxidant following drug treatment. The results shown in Fig. 7B reveal that treatment with either STS or rotenone caused a reduction in the intracellular levels of glutathione of N. crassa conidia. Interestingly, treatment with both STS and rotenone results in an increased depletion of intracellular glutathione. Glutathione efflux and intracellular depletion, besides the antioxidant properties of glutathione, appear to have an important role in cell death (21). Thus, the observed synergistic effect on intracellular glutathione depletion following exposure of N. crassa to STS and rotenone likely accounts for the synergistic effect of the drug combination on fungal cell death.

**DISCUSSION**

The protein kinase inhibitor staurosporine has been shown to induce programmed cell death in a variety of organisms and...
cell types (5, 12, 15, 24). It is able to activate a mitochondrion-dependent form of PCD, which includes ROS generation (24, 35, 45), although the role of ROS in the process of PCD remains controversial. In this study, we show that STS induces an apoptosis-like cell death in *N. crassa*, including a significant increase in ROS production that occurs in a dose- and time-dependent manner (Fig. 1 and 4). The antioxidant glutathione or its precursor N-acetylcysteine prevents ROS formation and also completely reverts the effects of STS on *N. crassa* (Fig. 5). These data support the hypothesis that ROS are important for STS-induced PCD. Furthermore, the inability of other tested antioxidants (β-carotene and ascorbic acid) to revert the effects of STS on cell growth was correlated with their inability to prevent STS-induced ROS production. Thus, we believe that an initial increase in ROS production is an important signaling event in PCD, as was previously proposed for different systems (24, 34, 39, 55).

Recent work has indicated that glutathione efflux by multidrug resistance-associated proteins (28) and the resulting cellular glutathione depletion, independent of ROS formation, are critical triggers of STS-induced PCD (12, 22). It was also observed that glutathione depletion is required for ROS formation (22). Our results are in agreement with these findings, pointing to an important role of glutathione depletion in drug-induced fungal cell death (also see below). The addition of glutathione could counteract glutathione loss (and also hamper ROS generation) and thus prevent the induction of *N. crassa* cell death by STS. In addition, the *nuo78* complex I mutant produces low levels of ROS upon STS exposure and yet is affected by the drug to an extent similar to that for the wild-type strain (Fig. 2 and 4).

A novel finding from this work is that selected complex I...
mutants are highly sensitive to STS, further confirming the known involvement of complex I in different PCD processes. This is the case for mutants lacking the 9.8-, 14-, 30.4-, and 51-kDa polypeptide subunits (Fig. 2). Complex I has been implicated in cell death induced by human viruses (33, 53) or other stimuli in both human and fungal cells (8, 31, 34, 39). Specific subunits of mammalian complex I have also per se been implicated in cell death. GRIM-19 (homologue of N. crassa NUO14 [41]) regulates cell death by binding a cytomegalovirus RNA (53) and is also involved in beta interferon- and retinoic acid-induced cancer cell death (31). Caspase cleavage of NDUFS1 (54) and cleavage of NDUFS3 by the granzyme A protease (42) were described to mediate death of human cells. These proteins are the homologues of N. crassa NUO78 (29) and NUO30.4 (60), respectively. Specific downregulation of NDUFA6 (homologue of N. crassa NUO14.8 [41]) but not other complex I subunits induces apoptosis in cells infected with HIV-1 (33). With the possible exception of NDUFA6, the other human complex I subunits were described as proapoptotic. We speculate that some complex I subunits, like NUO9.8, NUO14, NUO30.4, and NUO51, have an antia apoptotic role during STS-induced death of N. crassa. The fact that glutathione prevents STS effects on these mutants suggests that the protective role of these proteins might occur at a late stage of STS-induced cell death. This is in line with the suggestion that complex I affects late rather than early responses in signaling and gene expression induced by beta interferon and retinoic acid (31).

An important finding of this work is that treatment of N. crassa cells with rotenone, a classical inhibitor of respiratory chain complex I, enhances the antifungal properties of staurosporine (Fig. 3). While we were motivated to do the experiments by the findings that certain complex I mutants are very sensitive to STS, we also showed that the rotenone effect is independent of complex I inhibition. In fact, the synergistic effect was not observed when rotenone was substituted with other specific (piericidin A) or rather unspecific (DPI) inhibitors of complex I (Table 1). Conversely, the synergistic effect was observed both in N. crassa mutants with a nonfunctional complex I (Fig. 5) and in S. cerevisiae (Fig. 6), an organism that lacks complex I (49). These results corroborate a recent conclusion that inhibition of complex I is not required for cell death induced by rotenone, which is a model system for the study of parkinsonism (11). The authors found that dopaminergic mouse neurons carrying a deletion of a complex I subunit required for enzyme assembly and function were not protected from rotenone-induced death, as would be expected if rotenone acted by inhibiting complex I.

FIG. 7. Rotenone does not induce ROS production but enhances STS-driven glutathione depletion. (A) Overlay of green fluorescence histograms obtained for wild-type cells incubated for 60 min in the presence (gray) of 12.5 μM staurosporine (left), 100 μg/ml rotenone (ROT) (middle), or both (right). The production of ROS was analyzed by flow cytometry as detailed in Materials and Methods. The black lines represent results from control experiments with no additions. The percentages of cells producing ROS are indicated. (B) Total glutathione content in N. crassa cells previously grown for 5 h at 30°C, followed by growth for 1 h in the presence of the vehicle DMSO (white), 12.5 μM staurosporine (light gray), 100 μg/ml rotenone (dark gray), or both staurosporine and rotenone (black). The results represent the averages ± standard deviations from three independent experiments.
While rotenone has been described to induce cell death through the production of ROS (34, 39), this appears not to be the case with N. crassa. We could not detect ROS production upon treatment of the fungal cells with rotenone (Fig. 7A), presumably because N. crassa contains alternative NADH dehydrogenase enzymes (7, 16, 58) able to prevent ROS formation. A similar situation was observed in rat models of Parkinson’s disease, whereas transfection with a yeast NADH dehydrogenase prevented the overproduction of ROS induced by rotenone (39, 40). Recently, rotenone was described to stimulate ROS, protein carbonylation (61), and protein nitration, which was suggested to be a general signaling event underlying the formation of cytotoxic protein aggregates, such as the Lewy bodies characteristic of Parkinson’s disease (62). Rotenone also induces glutathione depletion (61; this work). These observations provide an attractive hypothesis to explain our results. In fact, we showed that the combination of rotenone and STS has a synergistic effect both on intracellular glutathione depletion (Fig. 7B) and on fungal cell death (Fig. 2 and 3) and that glutathione or its precursor N-acetylcysteine can revert the combined drug effects on N. crassa cells (Fig. 5). The fact that the two drugs act in synergy suggests that they induce different mechanisms of glutathione depletion.

Finally, we showed that rotenone also augments the activity of the death inducer staurosporine against other fungi, like S. cerevisiae, A. fumigatus, and C. albicans (Fig. 3 and 6). The last two represent a threat, especially to immunocompromised individuals (6). On the other hand, programmed cell death and tumor development are tightly linked phenomena. Modulation of cell death pathways is being explored to fight against cancer tumor development are tightly linked phenomena. Modulation of cell death pathways is being explored to fight against cancer.
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