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Involvement of mitochondrial proteins in calcium signalling and cell death induced by staurosporine in *Neurospora crassa*

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ABSTRACT

Staurosporine-induced cell death in *Neurospora crassa* includes a well defined sequence of alterations in cytosolic calcium levels, comprising extracellular Ca^{2+} influx and mobilization of Ca^{2+} from internal stores. Here, we show that cells undergoing respiratory stress due to the lack of certain components of the mitochondrial complex I (like the 51 kDa and 14 kDa subunits) or the Ca^{2+}-binding alternative NADPH dehydrogenase NDE-1 are hypersensitive to staurosporine and incapable of setting up a proper intracellular Ca^{2+} response. Cells expressing mutant forms of NUO51 that mimic human metabolic diseases also presented Ca^{2+} signalling deficiencies. Accumulation of reactive oxygen species is increased in cells lacking NDE-1 and seems to be required for Ca^{2+} oscillations in response to staurosporine. Measurement of the mitochondrial levels of Ca^{2+} further supported the involvement of these organelles in staurosporine-induced Ca^{2+} signalling. In summary, our data indicate that staurosporine-induced fungal cell death involves a sophisticated response linking Ca^{2+} dynamics and bioenergetics.

1. Introduction

An outstanding variety of intracellular organelles are able to accumulate Ca^{2+} including the Golgi apparatus, endosomes, secretory granules, lysosomes and nuclei though the classical Ca^{2+} reservoirs are the endoplasmic reticulum (ER), vacuoles and mitochondria [1]. Recent investigations shed light on the molecular identity of the machinery responsible for the transport of Ca^{2+} into and from the mitochondria (reviewed in [2]). In mammalian cells, accumulation of Ca^{2+} by the mitochondrial matrix occurs mainly through the calcium uniporter (MCU) at the inner mitochondrial membrane [3,4], although there is some evidence indicating the presence of supplementary molecules with the ability to move Ca^{2+} into the mitochondria, namely the mitochondrial ryanodine receptor type 1 [5], leucine zipper-EF-hand containing transmembrane protein 1 [6], and coenzyme Q10 [7]. The voltage-dependent anion-selective channels (VDACs) are involved in Ca^{2+} transport across the outer mitochondrial membrane. More specifically, VDAC1 seems to be accountable for the mitochondrial import of cell death-related Ca^{2+} signals from the ER [8]. Ca^{2+} release from the mitochondria may occur through the Na^{+}/Ca^{2+} exchanger (mNCX) [9] or the unspecific aperture known as the permeability transition pore [10]. In the fungus *Neurospora crassa*, the importance of mitochondria as Ca^{2+} stores is supported by the evidence that the accumulation of Ca^{2+}-containing mitochondria at the tip of hyphae is significant for the maintenance of a Ca^{2+}-gradient required for cell growth [11].

Apart from Ca^{2+} storage, mitochondria are well-known for their role in energy production. In most eukaryotes, energy production occurs at the inner mitochondrial membrane following the activity of specialized complexes of proteins (complexes I, II, III and IV) that comprise the electron transport chain. The proton pumping activity of these oligomeric complexes (except complex II) generates an electrochemical gradient used by the ATP synthase (complex V) to produce ATP [12]. In some fungi, plants, protists and bacteria, the electron transport chain is branched into single peptide alternative systems without proton translocation activity. The alternative oxidase (AOX) constitutes a detour for complexes III and IV whereas type II NAD(P)H dehydrogenases bypass complex I [13]. Alternative NAD(P)H dehydrogenases are particularly important not only because they oxidize NAD(P)H and reduce quinone but also because they serve as entry points for electrons into the respiratory chain [14–16]. Their importance is firmly demonstrated in *Saccharomyces cerevisiae*, where complex I is absent [17] and type II NAD(P)H dehydrogenases are the only existing NAD(P)H oxidases [18,19]. In *N. crassa*, four alternative rotenone-insensitive NAD(P)H dehydrogenases associated with the inner mitochondrial membrane have been characterized in addition to complex I [14,15]. NDI-1 [20] is localized at the matrix side of the membrane (internal enzyme), whereas NDE-1 [21], NDE-2 [22] and NDE-3 [23] are facing the intermembrane

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space (external enzymes). NDE-1 stands out because of its NADPH selectivity and regulation by pH and Ca^{2+} [24], being equivalent to the external NDB-1 of plants in this respect [25,26].

A rise in the intracellular levels of Ca^{2+} is a common cellular signalling mechanism in response to cell death stimuli. We showed recently that treatment of *N. crassa* cells with the cell death inducer staurosporine promotes a precise sequence of cytosolic Ca^{2+} transients (termed the staurosporine-induced Ca^{2+}-signature) fed by the release of Ca^{2+} from internal stores as well as by the influx from the extracellular milieu [27]. This is regulated through phospholipase C and involves activation of a cell membrane transient receptor potential (TRP)-like channel. Here we investigated the contribution of the mitochondria bioenergetics system for the development of the staurosporine-induced Ca^{2+}-signature. Our data revealed that components of the NADH:ubiquinone oxidoreductase (complex I) and at least one alternative NAD(P)H dehydrogenase (NDE-1) are crucial mediators of the fungal response to cell death and associated Ca^{2+} dynamics.

2. Material and methods

2.1. Strains, culture media and chemicals

N. crassa was handled according to standard procedures. Vogel's minimal medium plus 1.5% (w/v) sucrose was used in all experiments [28]. Wild type and other strains were obtained from the Fungal Genetics Stock Center [29] or previously generated in the laboratory like the deletion strains for alternative NAD(P)H dehydrogenases [20,22–24], Δ*nuo51* [30] *nuo51* rescued strain, *nuo51* A353V, *nuo51* T435M [31], Δ*nuo14* [32], Δ*nuo78* [33] and Δ*nuo24* [34]. The following chemicals were used during this study: staurosporine (LC Laboratories), dimethyl sulfoxide (DMSO), rotenone, oxaloacetic acid, antimycin A, potassium cyanide, oligomycin, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), diphenyleneiodonium (DPI), reduced glutathione (GSH) and N-acetylcysteine (NAC) (Sigma-Aldrich), thapsigargin, 1,2-bis(orthoaminophenoxy)ethane-N,N,N′,N′-tetrasodium (BAPTA) and Ru360 (Merck Millipore) and bafilomycin A1 (Wako Chemicals).

2.2. Intracellular Ca^{2+} measurement with aequorin

An aequorin-based luminescence method for the measurement of cytosolic Ca^{2+} optimized for filamentous fungi [35] was employed as described [27]. Briefly, aequorin-expressing conidia (transformed by electroporation with an Eppendorf Multiporator at 1800 V, 5 ms) at a concentration of 2×10^6 cells/ml were incubated in white opaque 96-well plates (100 μl/well) containing minimal medium with 5 μM coelenterazine (Santa Cruz Biotechnology) for 6 h at 26 °C, in the dark, without agitation. After treatment with the indicated drugs, luminescence (RLU, relative light units) of triplicates to hexaplicates was captured over time on a Bio-Tek Synergy HT microplate reader. Extra wells were prepared in order to check total emitted luminescence of each strain in each experiment using 100 μl of 3 M $CaCl_2$ in 20% ethanol. Total luminescence was used to normalize the experimental RLU. The values from control DMSO samples were subtracted from staurosporine-treated samples to obtain the "staurosporine-induced amplitude of response". Quantifications were obtained by summing the normalized experimental values. When specified, a pre-incubation step of 15 min with a pharmacological agent was applied before staurosporine. In all instances, the volume of chemical added to the wells was 10 μl (from an appropriate stock solution).

2.3. Expression of aequorin in mitochondria

A 207 bp cDNA fragment corresponding to the N-terminal mitochondria-targeting sequence of subunit 9 of *N. crassa* ATP synthase (NCU02250) was amplified by PCR using primers AACTAGTATGGCCTCCACTCGTGTCCTC and CCCCGGGGGAAGAGTAGGCGCGCTTCTG (introducing the underlined *Spe*I and *Sma*I sequences, respectively) and cloned in the pCRII-TOPO vector (Invitrogen). The *aequorin* gene was cloned into the *Sma*I restriction site (in front of the mitochondrial presequence) following its amplification from the pAB19 plasmid [36] by PCR using primers TCCCGGGATGACCTCCAAGCAGTACTCC and CCCGGGTTAATTAATTAGGGGACGGCACCGCCGTA. The aequorin gene fused to the mitochondrial presequence (*mitoAeq*) was amplified by PCR using primers CTGGCCGTCGTTTTAC and ATAGGATCCTTAGGGGACGGCA (introducing the underlined *Bam*HI sequence) and cloned back into pCRII-TOPO. After verification that the DNA sequence was correct, *mitoAeq* was excised from pCRII-TOPO using *Spe*I and *Bam*HI and cloned in the *N. crassa* pMF272 expression vector. The *N. crassa* his strain (FGSC #6103) was transformed by electroporation with an Eppendorf Multiporator at 1800 V for 5 ms.

The correct targeting of the mitoAeq fusion protein to mitochondria was verified by Western blot. *N. crassa* hyphae were homogenized with a pestle and mortar using quartz sand and mitochondrial isolation buffer (0.44 M sucrose, 2 mM EDTA, 30 mM Tris–HCl pH 7.6). Crude mitochondria and cytosol extracts were obtained by differential centrifugation as previously described [37] with an extra step of washing of the mitochondrial pellet. After denaturation at 95 °C for 5 min, 50 μg of the mitochondrial and cytosolic protein extracts were separated by SDS-PAGE using Criterion SFX 4–20% gels (Bio-Rad) and transferred to a nitrocellulose membrane. The membrane was blocked for 1 h using 5% non-fat dry milk and immunoblotted using an anti-aequorin antibody (Acris Antibodies). Proteins were detected by chemiluminescence using ChemiDoc (Bio-Rad).

2.4. Mitochondrial Ca^{2+} uptake

Reactions of 250 μl containing 200 μg mitochondria, 5 mM glutamate, 5 mM malate, 1 μM calcium green-5N (Life Technologies) in KCl media (125 mM KCl, 2 mM K_2HPO_4, 1 mM $MgCl_2$, 20 mM HEPES, pH 7.4) were incubated for 1 min at 26 °C in opaque 96-well plates. $CaCl_2$ was injected into the wells to a final concentration of 50 μM and emission of fluorescence was followed over time using a Bio-Tek Synergy HT microplate reader (excitation: 485/20 nm; emission: 528/20 nm). When indicated, 1 μM Ru360 or 4 μM CCCP or 0.5 mM BAPTA was added. The difference in fluorescence after the addition of 50 μM $CaCl_2$ was calculated for quantification purposes.

2.5. Other assays

For the spot growth assay, nine successive three-fold dilutions were prepared for each strain starting with 6.6×10^7 cells/ml. Five μl from each dilution was spotted on plates containing glucose–fructose–sorbose (GFS) medium with agar (to produce compact conidia) supplemented with the indicated chemicals. Cells were incubated at 26 °C and pictures taken ~72 h after inoculation. For the measurement of reactive oxygen species (ROS), conidia at 2×10^6 cells/ml were grown for 4 h in minimal medium at 26 °C. Dihydrorhodamine 123 (Sigma-Aldrich) at 20 μg/ml and staurosporine were then added during 30 min. Samples were harvested by centrifugation and washed twice with PBS before running on a BD FACS Calibur flow cytometer. Data were analyzed with FlowJo (Tree Star).

2.6. Statistical analysis

At least three independent experiments were performed in all instances and quantifications are expressed as mean ± SEM. The non-parametric Mann–Whitney test was used for comparisons between two groups using SPSS 20 (SPSS Inc.). p-values ≤ 0.05 were considered statistically significant.

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