

Subunits Rip1p and Cox9p of the respiratory chain contribute to diclofenac-induced mitochondrial dysfunction

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The widely used drug diclofenac can cause serious heart, liver and kidney injury, which may be related to its ability to cause mitochondrial dysfunction. Using *Saccharomyces cerevisiae* as a model system, we studied the mechanisms of diclofenac toxicity and the role of mitochondria therein. We found that diclofenac reduced cell growth and viability and increased levels of reactive oxygen species (ROS). Strains increasingly relying on respiration for their energy production showed enhanced sensitivity to diclofenac. Furthermore, oxygen consumption was inhibited by diclofenac, suggesting that the drug inhibits respiration. To identify the site of respiratory inhibition, we investigated the effects of deletion of respiratory chain subunits on diclofenac toxicity. Whereas deletion of most subunits had no effect, loss of either Rip1p of complex III or Cox9p of complex IV resulted in enhanced resistance to diclofenac. In these deletion strains, diclofenac did not increase ROS formation as severely as in the wild-type. Our data are consistent with a mechanism of toxicity in which diclofenac inhibits respiration by interfering with Rip1p and Cox9p in the respiratory chain, resulting in ROS production that causes cell death.

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INTRODUCTION

Diclofenac, a non-steroidal anti-inflammatory drug (NSAID), is widely used in the treatment of diseases involving pain and inflammation, such as arthritis. NSAIDs are inhibitors of prostaglandin synthesis and have been tested as anti-fungal agents, because *Candida albicans* and *Cryptococcus neoformans* secrete prostaglandin-like molecules involved in pathogenicity (Alem & Douglas, 2004; Noverr *et al.*, 2001). In humans, diclofenac causes serious adverse drug reactions that target the liver (Laine *et al.*, 2009), heart (Fosbøl *et al.*, 2009) and upper gastrointestinal tract (Lewis *et al.*, 2002). Mitochondrial dysfunction is increasingly considered a major cause of

drug-induced organ failure (Boelsterli & Lim, 2007; Dykens & Will, 2007). In mitochondria, electron-donating compounds such as NADH or succinate transfer electrons to the electron transport chain (ETC). The electrons travel through the respiratory chain complexes and are finally donated to oxygen, while protons are pumped across the membrane. This generates a proton motive force consisting of a membrane potential and a pH gradient that is used by ATPase to produce ATP. Various drugs can interfere with the electron transfer, lowering the mitochondrial membrane potential and decreasing the amount of ATP formed (Labbe *et al.*, 2008). Furthermore, the electrons can leak from the respiratory chain, generating reactive oxygen species (ROS) that can induce apoptosis via the mitochondrial permeability transition (MPT) (Nieminen *et al.*, 1997; Turrens, 2003).

There are several indications of a role for mitochondrial dysfunction in diclofenac toxicity. In rat hepatocytes, diclofenac causes uncoupling of mitochondrial respiration,

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; ETC, electron transport chain; MPT, mitochondrial permeability transition; NSAID, non-steroidal anti-inflammatory drug; PDR, pleiotropic drug resistance; pH_{cyt}, cytosolic pH; pH_{mit}, mitochondrial pH; ROS, reactive oxygen species.

in which oxygen consumption is no longer linked to proton transport over the inner membrane (Masubuchi *et al.*, 2000). This leads to decreased ATP production and ATP depletion (Bort *et al.*, 1999; Masubuchi *et al.*, 2002). Furthermore, in several cellular systems, including human hepatocytes, diclofenac induces ROS formation, causing successive opening of the MPT pore, cytochrome *c* release, caspase activation and apoptosis (Gómez-Lechón *et al.*, 2003; Inoue *et al.*, 2004; Lim *et al.*, 2006). However, the primary cause of the mitochondrial dysfunction remains unclear.

We used yeast as a model system to elucidate the mechanisms leading to mitochondrial dysfunction in diclofenac toxicity. Baker's yeast (*Saccharomyces cerevisiae*) is an excellent model organism to study mitochondrial functions, because a detailed knowledge of the composition and assembly of the various respiratory chain complexes can be combined with its genetic tractability. Furthermore, complexes II, III and IV are highly conserved among eukaryotes (Lemire & Oyedotun, 2002; Taanman & Capaldi, 1992; Zara *et al.*, 2009). Although yeast lacks complex I, the functional similarity of yeast NADH dehydrogenase Ndi1p allows rescue of NADH oxidase activity in complex I-deficient mammalian systems (Seo *et al.*, 1998).

Here, we clearly link diclofenac toxicity to respiration. We demonstrate the unique role of Rip1p in complex III and Cox9p in complex IV of the respiratory chain in the toxicity of diclofenac, and show that there is a strong correlation between diclofenac toxicity and the formation of ROS. These results indicate that diclofenac interferes with respiration at the site of Rip1p and Cox9p, leading to formation of ROS and cell death.

METHODS

Chemicals and stock solutions. Diclofenac was purchased as its sodium salt from Sigma Aldrich and dissolved in DMSO (500 mM). 2',7'-Dichlorofluorescein diacetate was obtained from Alexis Biochemicals and dissolved in ethanol (4 mM). All stock solutions were stored at -20°C and protected from light. All other chemicals were purchased from Sigma Aldrich at the highest purity.

Strains. The haploid BY4741 (MATa; his3 Δ 1; leu2 Δ 0; met15 Δ 0; ura3 Δ 0) wild-type and deletion strains were obtained from EUROSCARF. Strains without mitochondrial DNA (rho⁰) were generated by growth with ethidium bromide (10 $\mu\text{g ml}^{-1}$) for several days in rich medium [YPD: 1% (w/v) yeast extract, 2% (w/v) bactopectone, 2% (w/v) glucose]. Individual colonies were streaked in parallel on YPD and YPEG [1% (w/v) yeast extract, 2% (w/v) bactopectone, 3% (v/v) ethanol, 3% (v/v) glycerol]. Cells of the rho⁰ strain grow only on YPD plates (Goldring *et al.*, 1970). Loss of mitochondrial DNA was confirmed by staining the DNA with 4',6-diamidino-2-phenylindole (DAPI).

Construction of rescue constructs. *RIP1* and *COX9* genes including 500 bp up- and downstream were amplified by PCR and cloned into YCplac111 (pLEU), a yeast single-copy vector containing the CEN4/ARS1 origin of replication and a *LEU2* marker (Gietz & Sugino, 1988). The plasmids were transformed into the corresponding deletion strains by using the freeze-thaw method (Klebe *et al.*, 1983). Transformed strains were streaked in duplicate on YNB-Leu [0.67% (w/w) yeast nitrogen base without amino acids, 2% (w/w) glucose,

supplemented with amino acids and nucleotides without leucine] and YPEG. The Δcox9 and Δrip1 strains can grow only on YPEG when the deleted gene is complemented by the corresponding rescue construct.

Growth conditions and diclofenac treatment. Strains were grown overnight on minimal medium (YNB) at 30°C . Overnight cultures were diluted in minimal medium and grown at 30°C to OD₆₀₀ ~0.2. At this point, 0–100 μM diclofenac was added. Controls were treated with equal amounts of DMSO (maximum 0.1%, v/v). At various time points, the OD₆₀₀ of control and diclofenac-treated cultures was measured (Amersham Novaspec II spectrophotometer).

Measurement of cytosolic and mitochondrial pH (pH_{cyt} and pH_{mit}). pH_{cyt} and pH_{mit} were measured as described previously (Orij *et al.*, 2009). Briefly, strains containing a plasmid carrying the pHluorin gene with or without a mitochondrial targeting sequence were grown in black, clear-bottomed 96-well plates in minimal medium buffered at pH 5.0 with 25 mM sodium citrate. Fluorescence ($\lambda_{\text{ex}}=390$ or 470 nm, $\lambda_{\text{em}}=512$ nm) was measured and corrected for the cell density in the wells. The ratio of pHluorin emission intensity resulting from excitation at 390 and 470 nm was calculated and compared with a standard curve to determine the pH.

Oxygen consumption measurements. Oxygen consumption rates of whole cells were determined at 30°C with a Clark-type oxygen electrode. The reaction mixture (4 ml) contained 100 mM potassium phosphate buffer (pH 3.0 or 5.0), 10 mM MgSO₄ and $\sim 6 \times 10^6$ cells. Reactions were started by addition of 0.5% (w/v) glucose. After incubating for 2 min, 0–200 μM diclofenac was added. Oxygen-uptake rates were calculated based on a dissolved oxygen concentration of 236 μM in air-saturated water at 30°C .

Survival assay. Exponentially growing cultures of OD₆₀₀ ~0.2 were incubated with 0 or 100 μM diclofenac. After 6 h, the OD₆₀₀ was measured and 1×10^{-5} OD units (~300 cells) were plated on YPD plates. After incubation at 30°C for 3 days, the number of colonies was counted.

Measurement of ROS production. Exponentially growing cultures (2 ml) of OD₆₀₀ ~0.2 were treated with 0–50 μM diclofenac in the presence of 10 μM of the fluorescent, ROS-sensitive 2',7'-dichlorofluorescein diacetate. After 3 h at 30°C the cultures were centrifuged (3 min at 3000 r.p.m.), and cell pellets were washed and resuspended in 1 ml water. Fluorescence ($\lambda_{\text{ex}}=485$ nm, $\lambda_{\text{em}}=535$ nm) was measured and corrected for the cell density in the samples.

β -Galactosidase reporter assay. The *PDR3*- and *PDR5*-lacZ reporter constructs in the low-copy-number vector pSEYCI02 were a kind gift from Professor Scott Moye-Rowley, University of Iowa, Iowa City, IA, USA (Katzmann *et al.*, 1994). The plasmids were transformed into yeast by using the freeze-thaw method (Klebe *et al.*, 1983). Yeast cultures were grown at 30°C to OD₆₀₀ ~0.5. Protein extracts were made in LacZ buffer (40 mM Na₂HPO₄, 60 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, 50 mM β -mercaptoethanol) by using glass beads. Then, 5–35 μg protein was incubated with 24 μg chlorophenol red- β -D-galactopyranoside at 30°C for 10–360 min. *A*₅₇₅ was measured and corrected for the protein concentration in the samples and incubation time.

RESULTS

Respiring yeast cells show increased sensitivity to diclofenac

Previous studies in mammalian cells show that mitochondria play a role in diclofenac toxicity (Gómez-Lechón *et al.*, 2003; Inoue *et al.*, 2004; Lim *et al.*, 2006; Masubuchi *et al.*, 2002). To

investigate whether diclofenac interferes with mitochondrial respiration in *S. cerevisiae*, we studied the effect of various carbon sources on the sensitivity of wild-type cells to diclofenac. Growth of the cultures was monitored on medium containing glucose or galactose as carbon source supplemented with 0, 50 or 100 μM diclofenac (Fig. 1). When yeast is grown on glucose, the cells derive most of their energy from glycolysis. With galactose as carbon source, cells are more reliant on respiration (Fendt & Sauer, 2010). On glucose, cells treated with 50 μM diclofenac grew significantly more slowly than untreated cells (Fig. 1a). However, on galactose, cells grew even more slowly after treatment with 50 μM diclofenac (Fig. 1b). Clearly, when cells are increasingly dependent on respiration, diclofenac toxicity is enhanced. During incubation with 100 μM diclofenac in either glucose or galactose, cells barely grew at all.

To confirm that respiration increases diclofenac toxicity, we tested the diclofenac sensitivity of strains lacking mitochondrial DNA (ρ^0). Cells of the ρ^0 strains are deficient in respiration but can grow on fermentable carbon sources such as glucose, although more slowly than wild-type cells. Whereas wild-type cells hardly grew on 100 μM diclofenac, this diclofenac concentration had only a minor effect on the growth of ρ^0 cells (Fig. 1c), confirming the importance of respiration in diclofenac toxicity.

Diclofenac inhibits respiration but does not dissipate the proton gradient over the mitochondrial membrane

One of the current hypotheses regarding diclofenac toxicity is that it uncouples oxygen consumption from ATP production by dissipating the proton gradient (Masubuchi *et al.*, 2002). We used the pH-sensitive green fluorescent protein pHluorin (Oriji *et al.*, 2009) to measure pH_{cyt} and pH_{mit} . To rule out influences of external pH, cells were grown in medium buffered at pH 5. In this buffered medium, 200 μM diclofenac has similar effects on cell death and growth as 50 μM diclofenac in non-buffered medium (data not shown). As diclofenac is a weak acid ($\text{p}K_{\text{a}} \sim 4.0$), its

uptake is higher in the more acidic non-buffered medium (Wohnsland & Faller, 2001), leading to increased toxicity.

Wild-type cells were grown in glucose-containing media and fluorescence was monitored. The exponentially growing cells had a pH_{cyt} of 6.9 and a pH_{mit} of 7.2. After addition of 200 μM diclofenac, both values decreased quickly (Fig. 2a). After 30 min, the pH stabilized at 6.4 in the cytosol and 6.6 in the mitochondria and remained constant for at least 6 h (data not shown). Previously, the uncoupler carbonylcyanide *m*-chlorophenylhydrazone (CCCP) was shown to diminish the difference between pH_{cyt} and pH_{mit} (Oriji *et al.*, 2009). As the difference between mitochondrial and cytosolic pH remained intact with diclofenac, we conclude that diclofenac does not uncouple respiration.

As diclofenac is more toxic to respiring than to fermenting cells, we examined whether it inhibits respiration in yeast. Wild-type cells were incubated in potassium phosphate buffer at pH 3.0 or 5.0 and oxygen consumption was measured in the presence of glucose and 0–200 μM diclofenac (Fig. 2b). Oxygen consumption in the absence of diclofenac was comparable at both pHs (~ 7.4 fmol min^{-1} per cell) and was set at 100%. Diclofenac dose-dependently inhibited respiration at both pH 3.0 and 5.0. However, at pH 3.0 the inhibition was more severe. At pH 5.0, 200 μM diclofenac inhibited respiration by $\sim 13\%$, which is comparable with the inhibition by 50 μM diclofenac at pH 3.0. Because growth inhibition under these two conditions is comparable, there is a clear correlation between growth inhibition and reduced oxygen consumption at both pHs. Together, these results show that diclofenac inhibits, but does not uncouple, respiration.

Diclofenac induces the formation of ROS in wild-type but not in ρ^0 strains

As the respiratory chain is the main source of ROS in the mitochondria, we tested the effect of respiration on diclofenac-induced ROS formation. Wild-type and ρ^0

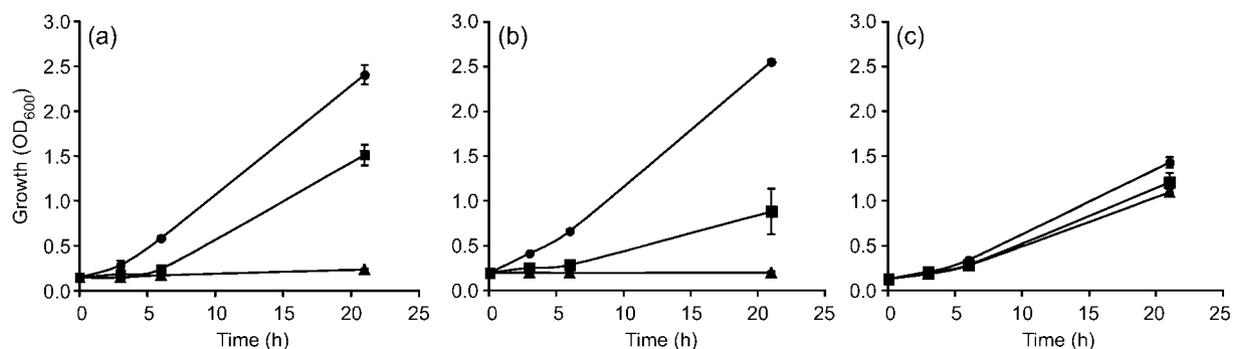


Fig. 1. Respiring cells are more sensitive to diclofenac. The wild-type yeast strain grown on (a) glucose or (b) galactose as carbon source and (c) a strain without mitochondrial DNA (ρ^0) grown on glucose were incubated with 0 μM (●), 50 μM (■) or 100 μM (▲) diclofenac. Growth is expressed as OD_{600} (mean \pm SD). Experiments were performed three times using independently generated ρ^0 strains.

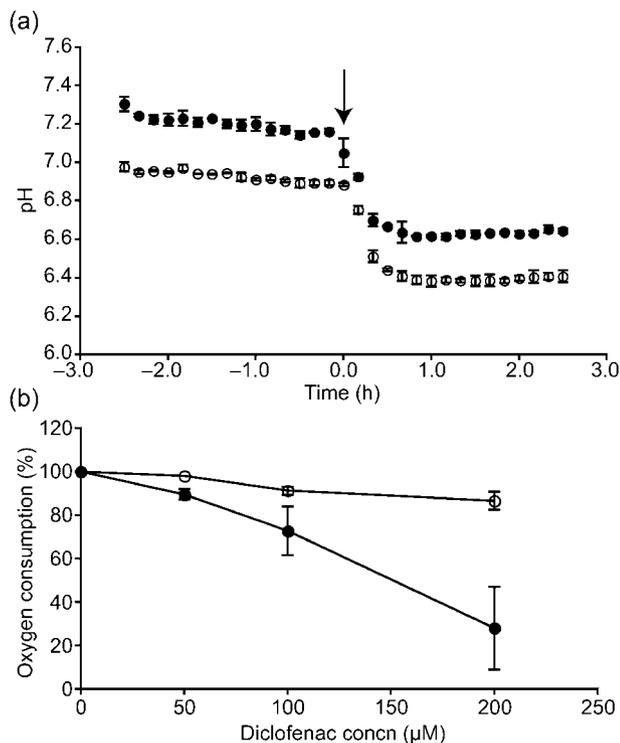


Fig. 2. Diclofenac inhibits oxygen consumption but does not influence the proton gradient. (a) Wild-type cells were grown in minimal media buffered at pH 5.0. pH_{cyt} (○) and pH_{mit} (●) were measured by using the pH-dependent fluorescent protein pHluorin. Fluorescence was corrected for cell density. At *t* = 0 (arrow), 200 μM diclofenac was added. Data are expressed as mean ± SD (*n* = 3). (b) Oxygen consumption was measured in wild-type cells incubated at pH 3.0 (○) or pH 5.0 (●) with 0–200 μM diclofenac. Data are expressed as percentage oxygen consumption compared with cells incubated without diclofenac (100 %) (mean ± SD, *n* = 3).

strains were incubated for 3 h in the presence of the ROS-sensitive fluorescent compound 2',7'-dichlorofluorescein diacetate and 0, 10, 30 or 50 μM diclofenac. ROS levels in the absence of diclofenac were set at 100%. In wild-type cells, diclofenac dose-dependently increased the amount of ROS formed (Fig. 3). Even at diclofenac concentrations of 30 μM the amount of ROS in the cells was doubled compared with wild-type cells incubated without diclofenac. At 50 μM diclofenac, cellular ROS levels were ~3.5-fold higher than in the untreated control cells. In contrast, these diclofenac concentrations did not induce ROS formation in the respiratory-deficient *rho*⁰ strain (Fig. 3) compared with *rho*⁰ controls. These results show a relationship between respiration, ROS formation and diclofenac toxicity.

Crucial role of respiratory chain subunits Rip1p and Cox9p in the toxicity of diclofenac

To investigate the role of mitochondrial respiration in diclofenac toxicity in more detail, we tested the effect of

individual respiratory chain proteins on diclofenac toxicity. Single gene deletion strains were used in which non-essential, nuclear-encoded subunits of the respiratory chain complexes were deleted. The deletion strains were grown for 6 h in the presence of 0 or 100 μM diclofenac. For each strain, the growth (increase in OD₆₀₀ in 6 h) of cultures incubated without diclofenac was set at 100% and the percentage growth of diclofenac-treated cultures was calculated (Fig. 4a).

Growth of wild-type cultures incubated with 100 μM diclofenac was only ~5% of that of control cultures. As also seen in Fig. 1, the *rho*⁰ strain was much more resistant to diclofenac, with ~80% growth compared with untreated cells. Deletion of the complex I homologue Ndi1p or subunits of complex II did not significantly change the sensitivity to diclofenac compared with wild-type cells. However, deletion of subunit Rip1p (Δ rip1) of complex III increased the resistance to diclofenac, with ~40% growth compared with control cells. Interestingly, deletion of subunit Cox9p (Δ cox9) of complex IV also increased diclofenac resistance.

The viability of the deletion strains after incubating for 6 h with 100 μM diclofenac was also analysed. The results (Fig. 4b) correlate with those of the growth assay. Only ~5% of the wild-type cells survived the diclofenac treatment. The *rho*⁰, Δ rip1 and Δ cox9 strains showed strongly increased resistance to diclofenac, with ~85, ~55 and ~65% of the cells surviving diclofenac treatment, respectively. Deletion of other complex III or IV subunits had some effect on diclofenac toxicity; in particular, the Δ cyt1 and Δ cox7 strains showed slightly increased diclofenac resistance in the growth and survival assays.

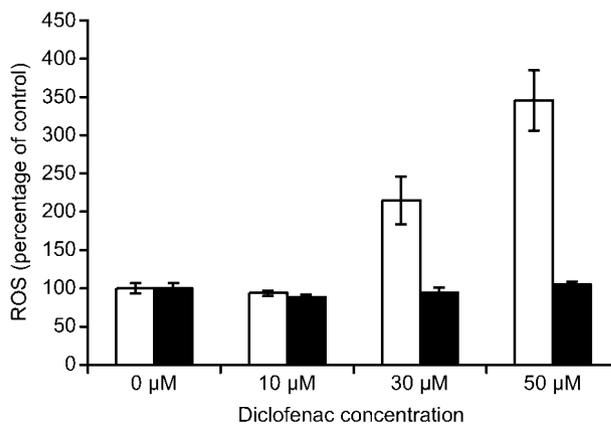


Fig. 3. Diclofenac increases ROS levels in wild-type but not in *rho*⁰ cells. The wild-type yeast strain (open bars) and a strain lacking mitochondrial DNA (*rho*⁰, closed bars) were grown in the presence of 0, 10, 30 or 50 μM diclofenac. After 3 h, ROS levels were measured by using the fluorescent ROS marker 2',7'-dichlorofluorescein diacetate. Measured fluorescence is corrected for cell density. ROS formation is expressed as percentage fluorescence compared with wild-type or *rho*⁰ controls incubated without diclofenac (100 %) (mean ± SD). Experiments were performed three times by using independently generated *rho*⁰ strains.

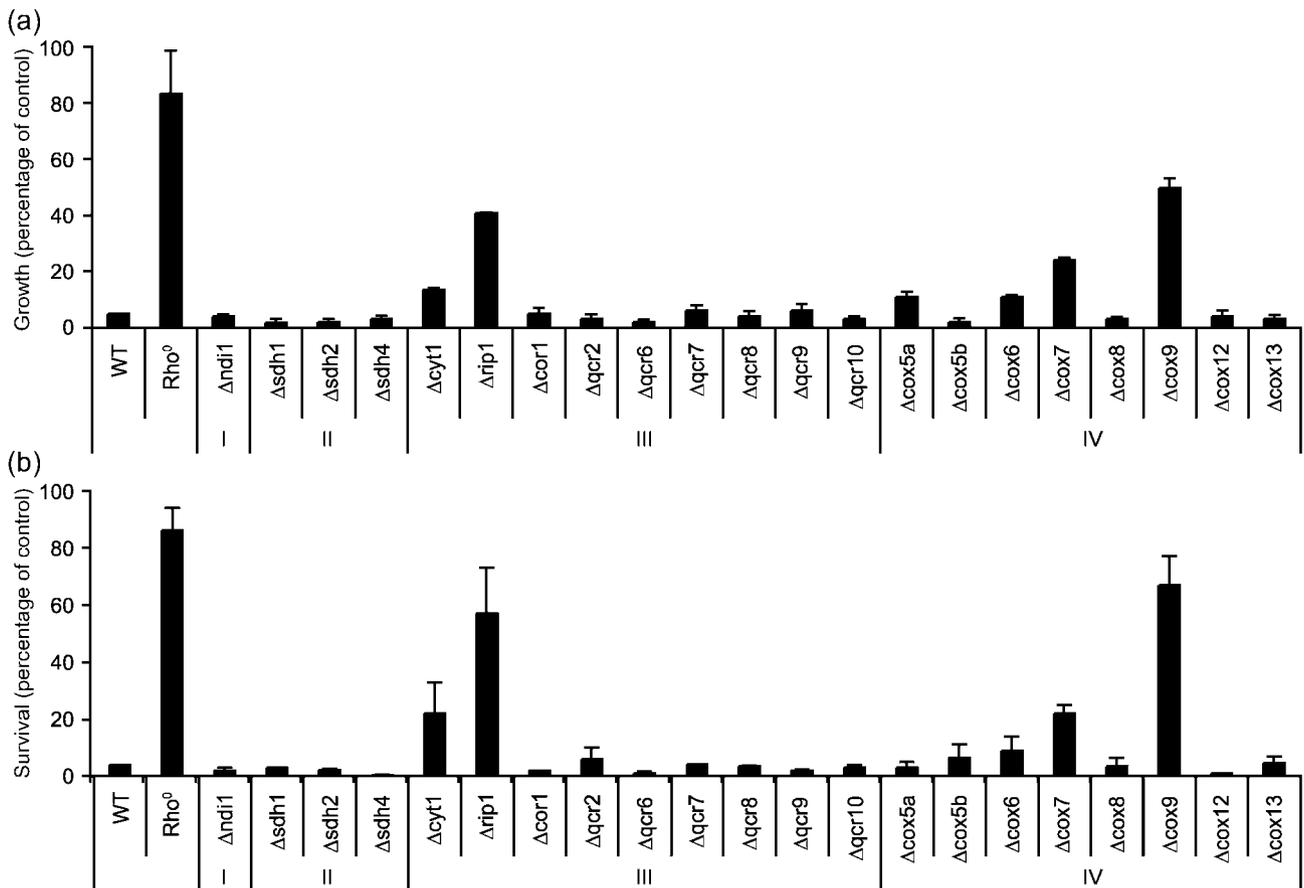


Fig. 4. Diclofenac toxicity is dependent on Rip1p and Cox9p. Growth (a) and survival (b) after incubation with 100 μ M diclofenac for 6 h of the wild-type strain (WT), a strain lacking mitochondrial DNA (Rho^0) and strains in which a subunit of respiratory chain complex I, II, III or IV was deleted. Data are expressed as percentage growth (OD_{600}) or percentage surviving cells compared with cells of the same strain incubated without diclofenac (100 %). Data represent means and SDs of two independent experiments performed in duplicate.

Complementation of the deleted genes restores diclofenac sensitivity

To confirm that the diclofenac resistance observed in the $\Delta rip1$ and $\Delta cox9$ deletion strains was solely due to absence of the *RIP1* and *COX9* genes, we introduced plasmid-based copies of the genes into the corresponding deletion strains. The *RIP1* and *COX9* genes were under the control of their own promoter. Successful complementation of the deleted gene should restore the ability to respire and result in a diclofenac-sensitive wild-type phenotype.

Wild-type, $\Delta rip1$ and $\Delta cox9$ strains transformed with an empty vector (pLEU), and the complemented strains $\Delta rip1 + pLEU-RIP1$ and $\Delta cox9 + pLEU-COX9$, were streaked in parallel on plates containing glucose (Fig. 5a) or ethanol and glycerol (Fig. 5b) as carbon source. All strains were able to grow on plates containing glucose. Whereas the $\Delta rip1$ and $\Delta cox9$ strains transformed with an empty vector were unable to grow on plates containing the non-fermentable carbon sources ethanol and glycerol, the

complemented $\Delta rip1 + pLEU-RIP1$ and $\Delta cox9 + pLEU-COX9$ strains grew on these plates. This shows that the ability to respire was restored in the complemented strains.

We also tested the effect of diclofenac on the growth of the strains. For each strain, growth without diclofenac was set at 100 %. Whereas the $\Delta rip1$ and $\Delta cox9$ strains were resistant to diclofenac, the $\Delta rip1$ and $\Delta cox9$ strains transformed with the pLEU-RIP1 and pLEU-COX9 constructs, respectively, were sensitive to diclofenac (Fig. 5c), confirming that Rip1p and Cox9p are involved in diclofenac toxicity.

Retrograde upregulation of the pleiotropic drug resistance (PDR) response is not involved in diclofenac resistance of complex III and IV deletion strains

In rho^0 cells and in strains lacking certain complex V subunits, several genes involved in PDR, including multidrug transporter *PDR5* and transcription factor *PDR3*, are

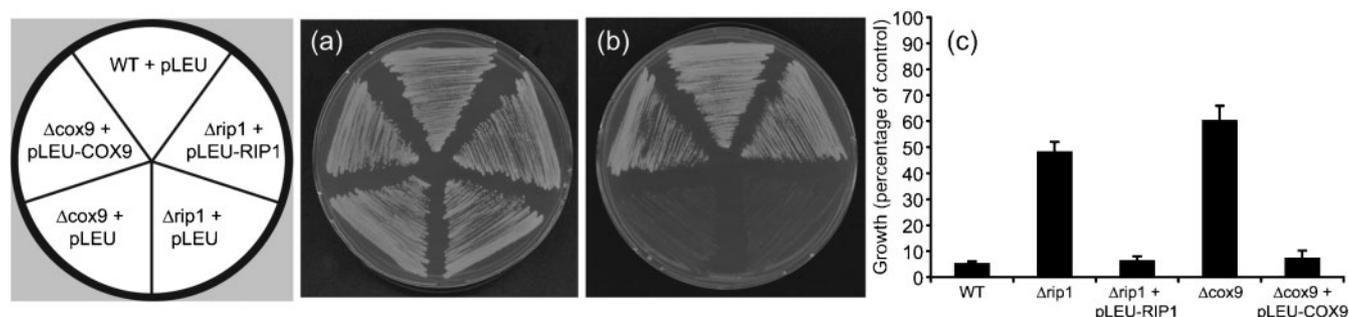


Fig. 5. Complementation of the deleted genes restores respiration and diclofenac sensitivity in $\Delta rip1$ and $\Delta cox9$ strains. The $\Delta rip1$ and $\Delta cox9$ deletion strains were transformed with constructs containing the *RIP1* and *COX9* genes under the control of their own promoter. Wild-type (WT), $\Delta rip1$ and $\Delta cox9$ strains transformed with an empty vector (pLEU) and the complemented $\Delta rip1 + pLEU-RIP1$ and $\Delta cox9 + pLEU-COX9$ were grown on plates containing (a) glucose (YPD) or (b) ethanol and glycerol (YPEG) as carbon source. Only strains that are able to respire can grow on YPEG plates. (c) Wild-type (WT), $\Delta rip1$, $\Delta cox9$ and the complemented $\Delta rip1 + pLEU-RIP1$ and $\Delta cox9 + pLEU-COX9$ strains were grown in the presence of 100 μM diclofenac. Data are expressed as percentage growth (OD_{600}) after 6 h compared with cells of the same strain incubated without diclofenac. Data represent means and SDs of two independent experiments performed in duplicate using different transformants.

upregulated compared with wild-type cells (Hallstrom & Moye-Rowley, 2000; Zhang & Moye-Rowley, 2001). Deletion of complex III or IV subunits may also cause upregulation of the PDR genes, which can affect diclofenac resistance. Therefore, we examined *PDR3* and *PDR5* expression in the diclofenac-resistant $\Delta rip1$ and $\Delta cox9$ strains. By using a β -galactosidase reporter assay, we measured *PDR3* and *PDR5* promoter activity. For both genes an increase in *lacZ* expression was observed in the ρ^0 strain (Table 1), as reported by Hallstrom & Moye-Rowley (2000). In the $\Delta rip1$ and $\Delta cox9$ strains, *lacZ* expression for both genes was similar to that in the wild-type. This indicates that diclofenac resistance in these deletion strains is not caused by an upregulation of the multidrug resistance genes.

Diclofenac sensitivity correlates with the formation of ROS

To explore further why deletion of Rip1p and Cox9p resulted in diclofenac resistance, whereas deletion of other

complex III or IV subunits did not, we investigated whether the deletion of these genes had an effect on the amount of ROS in the cell. First, we investigated ROS levels in the wild-type and deletion strains grown in the absence of diclofenac to compare basal ROS levels. The strains were grown for 3 h in the presence of the ROS marker 2',7'-dichlorofluorescein diacetate. No significant differences in basal ROS levels were observed (Fig. 6a).

Second, we tested ROS levels after incubation with 50 μM diclofenac and 2',7'-dichlorofluorescein diacetate for 3 h (Fig. 6b). In almost all deletion strains, ROS formation was increased approximately fourfold upon diclofenac treatment compared with ROS levels in the same strain incubated without diclofenac. Apparently, the disruption of stable complex III or IV formation does not decrease the potential of diclofenac to increase ROS formation. However, in the diclofenac-resistant $\Delta rip1$ and $\Delta cox9$ strains, ROS levels were only ~200 and ~150% of untreated controls, respectively. These results show a

Table 1. Multidrug resistance genes *PDR3* and *PDR5* are not upregulated in $\Delta rip1$ and $\Delta cox9$ strains

PDR3-lacZ and *PDR5-lacZ* expression in the wild-type strain, a strain lacking mitochondrial DNA (ρ^0) and strains in which either subunit Rip1p or Cox9p of the respiratory chain was deleted. Data are expressed as units of β -galactosidase activity, corrected for the protein concentration and incubation time. Data are means \pm SD of two experiments performed in duplicate using different transformants.

Strain	β -Galactosidase activity (units $\mu g^{-1} min^{-1}$)	
	<i>PDR3-lacZ</i>	<i>PDR5-lacZ</i>
Wild-type	1.7 \pm 0.3	77 \pm 11
ρ^0	3.4 \pm 0.4	213 \pm 22
$\Delta rip1$	2.1 \pm 0.1	87 \pm 27
$\Delta cox9$	1.8 \pm 0.2	90 \pm 21

strong correlation between diclofenac toxicity and ROS formation, and the unique roles of Rip1p and Cox9p within the ETC.

DISCUSSION

One of the challenges in examining the toxicity of drugs is identifying the cellular target(s). Yeast cells are useful in the identification of genes important in drug toxicity (Bharucha & Kumar, 2007). An advantage of yeast over mammalian cellular systems is the relatively straightforward way of performing genetic modifications. Here, we used yeast to study the mitochondrial toxicity of diclofenac.

In several cellular systems, including human hepatocytes, diclofenac can induce ROS formation that causes successive opening of the MPT pore, cytochrome *c* release,

caspase activation and apoptosis (Gómez-Lechón *et al.*, 2003; Inoue *et al.*, 2004; Lim *et al.*, 2006). As the respiratory chain is a well-known source of ROS (Kowaltowski *et al.*, 2009; Poyton *et al.*, 2009), we studied the role of respiration in diclofenac-induced ROS formation. We showed that when cells are increasingly dependent on respiration, diclofenac toxicity is enhanced (Fig. 1). Strains that lack mitochondrial DNA (ρ^0) are deficient in respiration and show an increased resistance to diclofenac (Fig. 1). Furthermore, loss of mtDNA completely diminished diclofenac-induced ROS formation (Fig. 3). These results indicate a clear link between mitochondrial respiration, ROS formation and diclofenac toxicity. Interestingly, another NSAID, aspirin, has also been linked to mitochondrial dysfunction in yeast, leading to apoptosis (Sapienza *et al.*, 2008).

Most of the oxygen consumed by yeast is used by the respiratory chain (reviewed by Rosenfeld & Beauvoit,

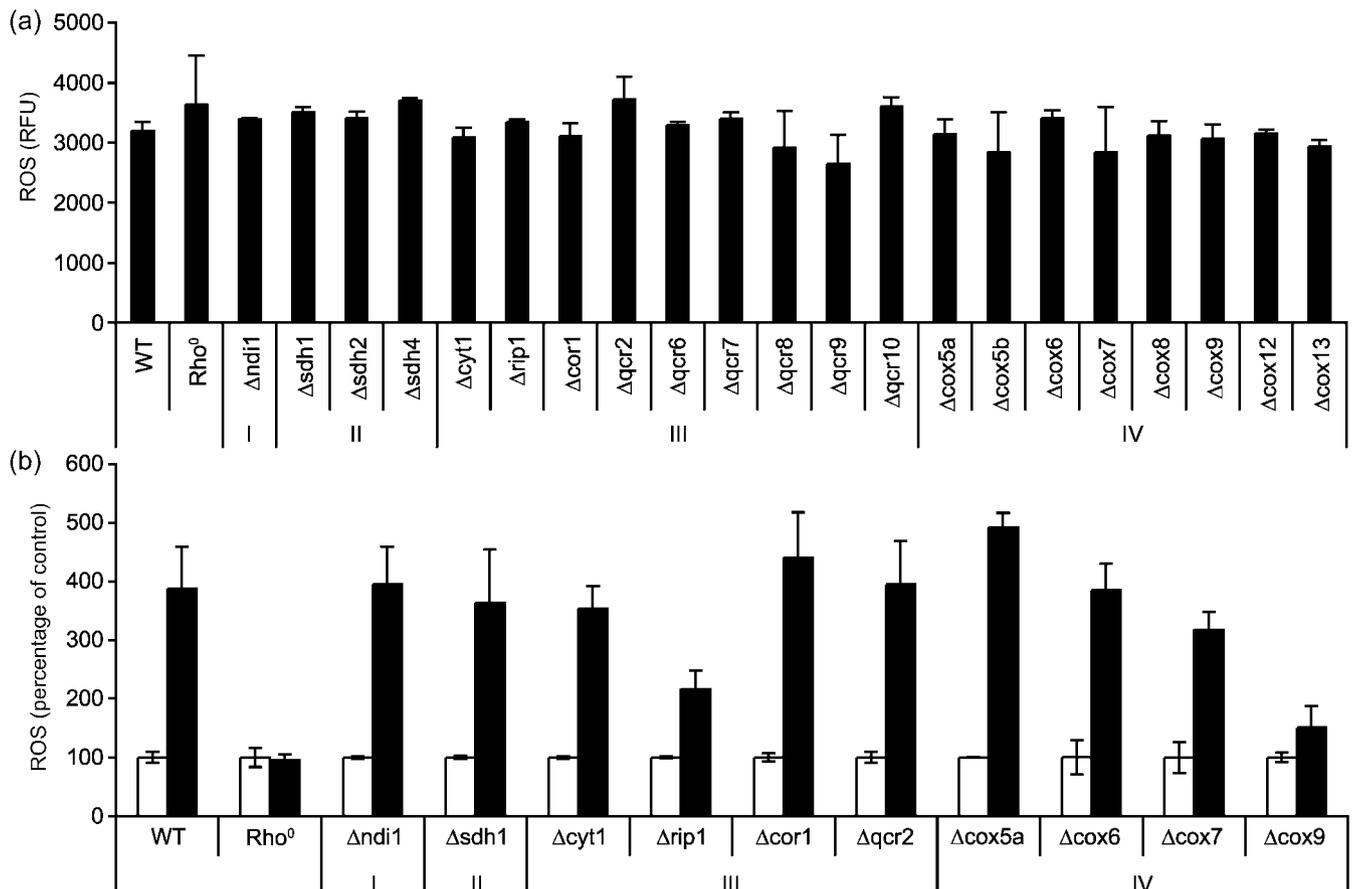


Fig. 6. Diclofenac resistance is correlated with ROS formation. (a) Basal ROS levels in the wild-type (WT), a strain lacking mitochondrial DNA (ρ^0) and strains in which a subunit of respiratory chain complex I, II, III or IV was deleted. For this experiment the strains were not incubated with diclofenac. (b) ROS formation after 3 h of incubation without (open bars) or with 50 μ M diclofenac (closed bars) in the wild-type (WT), a strain lacking mitochondrial DNA (ρ^0) and strains in which a subunit of respiratory chain complex I, II, III or IV was deleted. ROS formation was measured by using the fluorescent ROS marker 2',7'-dichlorofluorescein diacetate. Fluorescence is corrected for cell density and expressed as units (relative fluorescence units; RFU) or as a percentage compared with cells of the same strain incubated without diclofenac. Values are mean \pm SD ($n=4$).

2003). Respiratory inhibitors can almost fully inhibit oxygen consumption. Here, we show that diclofenac can also inhibit oxygen consumption in yeast. Inhibition of oxygen consumption by diclofenac was also found in mammalian cells (Niklas *et al.*, 2009; Krause *et al.*, 2003). Uncoupling of respiration by diclofenac is thought to be responsible for mitochondrial dysfunction. Although a collapse of the mitochondrial membrane potential after diclofenac exposure has been described in mammalian cells (Bort *et al.*, 1999; Inoue *et al.*, 2004; Lim *et al.*, 2006; Masubuchi *et al.*, 2002), this can also be the result of opening of the MPT pore instead of uncoupling. Here, we followed the effect of diclofenac on pH_{cyt} and pH_{mit} over time. We observed an immediate decrease in both pH_{cyt} and pH_{mit} , but the difference between the two values remained intact. Similar results have been described for the complex III inhibitor antimycin A, while the uncoupler CCCP does lead to dissipation of the pH difference (Orij *et al.*, 2009). This indicates that under these experimental conditions, diclofenac does not uncouple respiration or induce MPT pore opening in yeast.

Using single gene deletion mutants, we identified Rip1p and Cox9p of complex III and IV, respectively, as possible targets of diclofenac in the respiratory chain. Deletion of Rip1p or Cox9p markedly increased growth and survival during diclofenac treatment compared with wild-type and other deletion strains (Fig. 4). Furthermore, Δrip1 and Δcox9 strains had markedly lower ROS levels during diclofenac incubation than the wild-type strain (Fig. 6b). To determine ROS formation we used 2',7'-dichlorofluorescein diacetate. However, as this probe is slightly pH-sensitive we repeated the ROS assay for wild-type, Δrip1 and Δcox9 strains with another ROS-sensitive probe, dihydroethidium, and obtained similar results (data not shown). This strong correlation between ROS levels and diclofenac sensitivity indicates that the formation of ROS is the main cause of toxicity. The mitochondrial superoxide dismutase SOD2 has been suggested to be a diclofenac target in neuroblastoma cells, as evidenced by reduced levels of SOD2 protein and activity upon diclofenac exposure (Cecere *et al.*, 2010). Therefore, we tested deletion mutants of *SOD1* and *SOD2* in yeast, but did not observe a reduced diclofenac sensitivity compared with the wild-type over 21 h of incubation.

We confirmed the role of Rip1p and Cox9p in diclofenac toxicity by showing that complementation of the genes in the deletion strains restores the diclofenac-sensitive phenotype. Although the diclofenac resistance of Δrip1 and Δcox9 strains was significantly increased compared with wild-type cells, they were not as resistant as the rho^0 strain. The higher diclofenac resistance of the rho^0 cells can be explained as an additional effect of lacking both complex III and complex IV activity. Furthermore, upregulation of the PDR multidrug response in rho^0 cells (Table 1) (Hallstrom & Moye-Rowley, 2000) may contribute to the resistance. For other NSAIDs such as acetaminophen (Srikanth *et al.*, 2005) and indomethacin (Mima *et al.*,

2007), a role for ABC transporters in their toxicity towards *S. cerevisiae* has been shown.

Rip1p, a Rieske iron-sulfur protein, is one of the catalytic subunits of complex III. Complex III has two distinct binding sites for ubiquinone: the Qi site and the Qo site. The Qo site consists of the proximal niche close to the haem bL of the mitochondrially encoded cytochrome B (Cobp) and the distal niche close to the iron-sulfur cluster of Rip1p. In particular, complex III inhibitors that bind in the proximal Qo niche or at the Qi site induce ROS formation by inhibiting electron transport (Muller *et al.*, 2003; Yang *et al.*, 2008). It is tempting to speculate that diclofenac can bind at one of these sites, thereby inhibiting respiration and inducing leakage of electrons. In the absence of Rip1p, the ability of diclofenac to generate ROS is lost. Loss of the iron-sulfur protein also abolishes the increase in hypoxia- or methyl mercury-induced ROS (Guzy *et al.*, 2007; Lee *et al.*, 2009). Furthermore, inhibition of Rip1p mobility by stigmatellin decreases both basal and induced ROS formation (Armstrong *et al.*, 2004; Muller *et al.*, 2003). This indicates that Rip1p is crucial for ROS formation at complex III. Also, loss of Cyt1p slightly increased diclofenac resistance, which might be explained by the direct interaction of Cyt1p with Rip1p. Interestingly, *RIP1* is highly conserved across eukaryotes (Beckmann *et al.*, 1989), and downregulation of the mammalian homologue of Rip1p, RISP (UQCERS1), has also been shown to reduce ROS production (Guzy *et al.*, 2005).

Cox9p is a small (7 kDa) protein that is essential for complex IV activity (Wright *et al.*, 1986). In the literature, the protein encoded by *COX9* is also referred to as subunit VIIa, whereas the gene product of *COX12* is sometimes named Cox9p. Here, we use the nomenclature of the *Saccharomyces* Genome Database (<http://www.yeastgenome.org>) and refer to Cox9p as the gene product of *COX9*. We show that lack of subunit Cox9p lowers diclofenac-induced ROS formation. Cox9p has been suggested to play a role in complex IV assembly or stability (McEwen *et al.*, 1986). Possibly, interactions of diclofenac with Cox9p interfere with complex IV integrity, leading to ROS formation. Interestingly, complex IV has rarely been associated with ROS formation in the literature. As complexes III and IV assemble in a supercomplex, lack of Cox9p might influence electron transfer between the complexes, leading to ROS formation at complex III.

Surprisingly, deletion of several complex III and IV subunits (e.g. Cor1p and Cox6p) has been reported to cause loss of Rip1p and Cox9p from the complexes (Calder & McEwen, 1991; Crivellone *et al.*, 1988; Dowhan *et al.*, 1985) but does not result in diclofenac resistance or reduced ROS formation (Figs 4 and 6b). Perhaps in these cases, subcomplexes of complex III or complex IV are formed that are not stable enough for isolation and are therefore not detected.

In conclusion, we have demonstrated that subunits Rip1p of respiratory chain complex III and Cox9p of complex IV

are crucial to the mitochondrial toxicity of diclofenac. Further research is needed to see whether the mammalian counterparts UQCRCFS1 and COX6c (Lenaz & Genova, 2010) are related to diclofenac toxicity in mammalian cells and to understand how the two proteins dictate diclofenac interference at the ETC.

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