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

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Chapter 2

ABSTRACT

The widely used drug diclofenac can cause serious heart, liver or 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 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.

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 and Douglas, 2004; Noverr et al., 2001). In humans, diclofenac causes serious adverse drug reactions that target the liver (Laine et al., 2009) heart (Fosbol 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 and Lim, 2007; Dykens and Will, 2007). In mitochondria, electron donating compounds like 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, leading to lowering of 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).
Diclofenac targets Rip1p and Cox9p in the mitochondria

There are several indications for a role of mitochondrial dysfunction in diclofenac toxicity. In rat hepatocytes, diclofenac causes uncoupling of mitochondrial respiration, in which oxygen consumption is no longer linked to proton transport over the inner membrane (Masubuchi et al., 2000). This leads to a 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 (Gomez-Lechon 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 on the composition and assembly of the various respiratory chain complexes can be combined with its genetic tractability. Furthermore, complex II, III and IV are highly conserved among eukaryotes (Lemire and Oyedotun, 2002; Taanman and Capaldi, 1992; Zara et al., 2009). Although yeast lacks complex I, the functional similarity of yeast NADH dehydrogenase Nd1p 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.

MATERIALS AND METHODS

Chemicals and stock solutions
Diclofenac was purchased as its sodium salt form Sigma Aldrich and dissolved in DMSO (500 mM). 2’,7’-Dichlorodihydrofluorescein 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; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0) wild type and deletion strains were obtained from EUROSCARF. Strains without mitochondrial DNA (ρ06) were
generated by growth with 10 µg ml\(^{-1}\) ethidium bromide for several days in rich medium (YPD: 1% w/v yeast extract, 2% w/v bactopeptone, 2% w/v glucose). Individual colonies were streaked in parallel on YPD and YPEG (1% w/v yeast extract, 2% w/v bactopeptone, 3% v/v ethanol and 3% v/v glycerol). Rho\(^0\) cells grow only on YPD plates (Goldring et al., 1970). Loss of mitochondrial DNA was confirmed by staining the DNA with DAPI (4\'-6-diamidino-2-phenylindole).

**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 and 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 duplicates on YNB-Leu (0.67% w/w yeast nitrogen base without amino acids, 2% w/w glucose, supplemented amino acids and nucleotides without leucine) and YPEG. The ∆cox9 and ∆rip1 strains can only grow 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 OD\(_{600}\) ~0.2. At this point, 0-100 µM diclofenac was added. Controls were treated with equal amounts of DMSO (max 0.1% v/v). At various time points, the OD\(_{600}\) of control and diclofenac treated cultures was measured (Amersham Novaspec II spectrophotometer).

**Measurement of cytosolic and mitochondrial pH**

Cytosolic and mitochondrial pH 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 media 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 nm 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 MgSO\(_4\), and \(\sim6\times10^6\) cells. Reactions were started by addition of 0.5% w/v
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Diclofenac targets Rip1p and Cox9p in the mitochondria 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 M 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 hours, the OD600 was measured and 1x10^5 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’-dichlorodihydrofluorescein diacetate. After 3 hours at 30 °C the cultures were centrifuged (3 min 3000 rpm) and cell pellets were washed and resuspended in 1 ml water. Fluorescence (λex = 485 nm, λem = 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 pSEYC102 were a kind gift from Prof. Scott Moye-Rowley (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 chlorophenolred-β-D-galactopyranoside at 30 °C for 10-360 minutes. Absorption at 575 nm 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 and 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.

![Fig. 1.](image)

To confirm that respiration increases diclofenac toxicity, we tested the diclofenac sensitivity of strains lacking mitochondrial DNA (rho0). Rho0 cells 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 rho0 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 (Orij et al., 2009) to measure the cytosolic and mitochondrial pH (pH_{cyt} and pH_{mit} respectively). To rule out influences of external pH, cells were grown in media 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 (pKa ~4.0), its uptake is higher in the more acidic non-buffered medium (Wohnsland and 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 minutes, the pH stabilized at 6.4 in the cytosol and 6.6 in the mitochondria and remained constant for at least six hours (data not shown). Previously, the uncoupler CCCP was shown to diminish the difference between pH_{cyt} and pH_{mit} (Orij et al., 2009). As the difference between mitochondrial and cytosolic pH remained intact with diclofenac, we conclude that diclofenac does not uncouple respiration.

Since 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$^{-1}$ cell$^{-1}$) 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 ~13%, which is comparable with the inhibition by 50 µM diclofenac at pH 3.0. Because growth inhibition at 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 reactive oxygen species in WT but not in rho\(^0\) 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 rho\(^0\) strains were incubated for 3 hours in the presence of the ROS-sensitive fluorescent compound 2',7'-dichlorodihydrofluorescein diacetate and 0, 10, 30 or 50 \(\mu\text{M}\) diclofenac.

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**Fig. 3.** Diclofenac increases ROS levels in wild type, but not in rho\(^0\) cells. The wild type yeast strain (white bars) and a strain lacking mitochondrial DNA (rho\(^0\), black bars) were grown in the presence of 0, 10, 30 or 50 \(\mu\text{M}\) diclofenac. After 3 hours, the ROS levels were measured using the fluorescent ROS marker 2',7'-dichlorodihydrofluorescein diacetate. Measured fluorescence is corrected for cell density. ROS formation is expressed as % fluorescence compared with WT or rho\(^0\) controls incubated without diclofenac (100 %) \(\pm\) SD. Experiments were performed three times using independently generated rho\(^0\) strains.

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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, white bars). Even at diclofenac concentrations of 30 \(\mu\text{M}\) the amount of ROS in the cells was doubled compared with wild type cells incubated without diclofenac. At 50 \(\mu\text{M}\) diclofenac, the cellular ROS levels were \(\sim\)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, black bars) compared with rho\(^0\) controls. These results show a relationship between respiration, ROS formation and diclofenac toxicity.

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**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 hours in the presence of 0 or 100 \(\mu\text{M}\) diclofenac. For each strain, the growth (increase in OD\(_{600}\) in 6 hours) of cultures
Diclofenac targets Rip1p and Cox9p in the mitochondria

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<sup>0</sup> strain was much more resistant to diclofenac, with ~80% growth compared with untreated cells. Deletion of 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 (∆rip1) of complex III increased the resistance to diclofenac, with ~40% growth compared with control cells. Interestingly, deletion of subunit Cox9p (∆cox9) of complex IV also increased diclofenac resistance.

Fig. 4. Diclofenac toxicity is dependent on Rip1p and Cox9p. Growth (A) and survival (B) of the wild type strain (WT), a strain lacking mitochondrial DNA (rho<sup>0</sup>) and strains in which a subunit of respiratory chain complex I, II, III or IV was deleted after incubation with 100 µM diclofenac for 6 hours. The data are expressed as % growth (OD<sub>600</sub>) or % surviving cells ± SD compared with cells of the same strain incubated without diclofenac (100%).
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The viability of the deletion strains after incubating 6 hours with 100 μM diclofenac was also analyzed. The results (Fig. 4B) correlate with those of the growth assay. Only ~5% of the WT cells survived the diclofenac treatment. The rho\(^0\), Δrip1 and Δcox9 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 Δcyt1 and Δcox7 strains showed slightly increased diclofenac resistance in the growth and survival assays.

Fig. 5. Complementation of the deleted genes restores respiration and diclofenac sensitivity in Δrip1 and Δcox9 strains. The Δrip1 and Δcox9 deletion strains were transformed with constructs containing the RIP1 and COX9 genes under control of their own promoter. Wild type (WT), Δrip1 and Δcox9 strains transformed with an empty vector (pLEU) and the complemented Δrip1 + pLEU-RIP1 and Δcox9 + 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, Δrip1, Δcox9 and the complemented Δrip1 + pLEU-RIP1 and Δcox9 + pLEU-COX9 strains were grown in the presence of 100 μM diclofenac. The data are expressed as % growth (OD\(_{600}\)) ± SD after 6 hours compared with cells of the same strain incubated without diclofenac. Data represent means of two independent experiments performed in duplicate using different transformants.
Diclofenac targets Rip1p and Cox9p in the mitochondria

Complementation of the deleted genes restores diclofenac sensitivity
To confirm that the diclofenac resistance observed in the Δrip1 and Δcox9 deletion strains was solely due to absence of the RIP1 and COX9 genes, we introduced a plasmid-based copy of the genes into the corresponding deletion strains. The RIP1 and COX9 genes were under 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, Δrip1 and Δcox9 strains transformed with an empty vector (pLEU) and the complemented strains Δrip1 + pLEU-RIP1 and Δcox9 + 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 Δrip1 and Δcox9 strains transformed with an empty vector were unable to grow on plates containing the non-fermentable carbon sources ethanol and glycerol, the complemented Δrip1 + pLEU-RIP1 and Δcox9 + 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 Δrip1 and Δcox9 strains were resistant to diclofenac, the Δrip1 and Δcox9 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 PDR response is not involved in diclofenac resistance of complex III and IV deletion strains
In rho⁰ cells and in strains lacking certain complex V subunits, several genes involved in pleiotropic drug resistance (PDR), including multidrug transporter PDR5 and transcription factor PDR3, are upregulated compared with wild type cells (Hallstrom and Moye-Rowley, 2000; Zhang and 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 Δrip1 and Δcox9 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 rho⁰ strain (Table 1), as reported before by Hallstrom and Moye-Rowley (2000). In the Δrip1 and Δcox9 strains, lacZ expression for both genes was similar to that in wild type. This indicates that diclofenac resistance in these deletion strains is not caused by an upregulation of the multidrug resistance genes.
Table 1. Multidrug resistance genes *PDR3* and *PDR5* are not upregulated in ∆rip1 and ∆cox9 strains. *PDR3-lacZ* and *PDR5-lacZ* expression in the wild type strain, a strain lacking mitochondrial DNA (*rho*^0^) and strains in which either subunit Rip1p or Cox9p of the respiratory chain was deleted. Data are expressed as β-galactosidase activity in units, corrected for the protein concentration and incubation time, ± SD. Data are means of two experiments performed in duplicate using different transformants.

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<th>β-galactosidase activity (units µg⁻¹ min⁻¹)</th>
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<tr>
<td></td>
<td><em>PDR3-lacZ</em></td>
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<tr>
<td>Wild type</td>
<td>1.7 ± 0.3</td>
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<tr>
<td>Rho^0^</td>
<td>3.4 ± 0.4</td>
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<tr>
<td>∆rip1</td>
<td>2.1 ± 0.1</td>
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<tr>
<td>∆cox9</td>
<td>1.8 ± 0.2</td>
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Diclofenac sensitivity correlates with the formation of reactive oxygen species

To further explore 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 the ROS levels in the wild type and deletion strains grown in absence of diclofenac to compare the basal ROS levels. The strains were grown for 3 hours in presence of the ROS marker 2’,7’-dichlorodihydrofluorescein diacetate. No significant differences in basal ROS levels were observed (Fig. 6A).

Secondly, we tested ROS levels after incubation with 50 µM diclofenac and 2’,7’-dichlorodihydrofluorescein diacetate for 3 hours (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 ∆rip1 and ∆cox9 strains, ROS levels were only ~200% and ~150% of untreated controls, respectively. These results show a strong correlation between diclofenac toxicity and ROS formation, and the unique roles of Rip1p and Cox9p within the electron transfer chain.
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**Fig. 6.** Diclofenac resistance is correlated with ROS formation. (A) Basal ROS levels in wild type (WT), a strain lacking mitochondrial DNA (rho<sup>0</sup>) 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 in wild type (WT), a strain lacking mitochondrial DNA (rho<sup>0</sup>) and strains in which a subunit of respiratory chain complex I, II, III or IV was deleted after 3 hours of incubation without (white bars) or with 50 µM diclofenac (black bars). ROS formation was measured using the fluorescent ROS marker 2',7'-dichlorodihydrofluorescein diacetate. Fluorescence is corrected for cell density and expressed as units (RFU) or % compared with cells of the same strain incubated without diclofenac ± SD (n=4).

**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 and 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 reactive oxygen species (ROS) formation that causes successive opening of the mitochondrial permeability transition (MPT) pore, cytochrome c release, caspase activation and apoptosis (Gomez-Lechon et al., 2003, Inoue et al., 2004, Lim et al., 2006). Since 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 (rho⁰) 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 and Beauvoit, 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., 1998; 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 the cytosolic and mitochondrial pH over time. We observed an immediate decrease in both cytosolic and mitochondrial pH, 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’-dichlorodihydrofluorescein diacetate. However, as this probe is slightly pH sensitive we repeated the ROS assay for WT, 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
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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 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 IV activity. Furthermore, upregulation of the PDR multidrug response in rho^0 cells (Table 1) (Hallstrom and Moye-Rowley, 2000) may contribute to the resistance. For other NSAIDs like acetaminophen (Srikanth et al., 2005) and indomethacin (Mima et al., 2007), a role for ABC transporters on 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 heme 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 methylmercury-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 (UQCRFS1), 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 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 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 (for example Cor1p and Cox6p) has been reported to cause loss of Rip1p and Cox9p from the complexes (Calder and McEwen, 1991; Crivellone et al., 1988; Dowhan et al., 1985) but does not result in diclofenac resistance or reduced ROS formation (Fig. 4 and 6B). Perhaps in these cases subcomplexes of complex III or IV are formed that are not stable enough for isolation and are therefore not detected.

In conclusion, we 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 UQCRFS1 and COX6c (Lenaz and Genova, 2010) are related to diclofenac toxicity in mammalian cells and to understand how these two proteins dictate diclofenac interference at the ETC.

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REFERENCES


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Chapter 2


