Applied and Environmental Microbiology, Sept. 2011, p. 5973–5980 Vol. 77, No. 17
0099-2240/11/$12.00 doi:10.1128/AEM.00253-11

Involvement of the Pleiotropic Drug Resistance Response, Protein Kinase C Signaling, and Altered Zinc Homeostasis in Resistance of *Saccharomyces cerevisiae* to Diclofenac[▽]

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Received 4 February 2011/Accepted 25 June 2011

Diclofenac is a widely used analgesic drug that can cause serious adverse drug reactions. We used *Saccharomyces cerevisiae* as a model eukaryote with which to elucidate the molecular mechanisms of diclofenac toxicity and resistance. Although most yeast cells died during the initial diclofenac treatment, some survived and started growing again. Microarray analysis of the adapted cells identified three major processes involved in diclofenac detoxification and tolerance. In particular, pleiotropic drug resistance (PDR) genes and genes under the control of Rlm1p, a transcription factor in the protein kinase C (PKC) pathway, were upregulated in diclofenac-adapted cells. We tested if these processes or pathways were directly involved in diclofenac toxicity or resistance. Of the pleiotropic drug resistance gene products, the multidrug transporter Pdr5p was crucially important for diclofenac tolerance. Furthermore, deletion of components of the cell wall stress-responsive PKC pathway increased diclofenac toxicity, whereas incubation of cells with the cell wall stressor calcofluor white before the addition of diclofenac decreased its toxicity. Also, diclofenac induced flocculation, which might trigger the cell wall alterations. Genes involved in ribosome biogenesis and rRNA processing were downregulated, as were zinc-responsive genes. Paradoxically, deletion of the zinc-responsive transcription factor Zap1p or addition of the zinc chelator 1,10-phenanthroline significantly increased diclofenac toxicity, establishing a regulatory role for zinc in diclofenac resistance. In conclusion, we have identified three new pathways involved in diclofenac tolerance in yeast, namely, Pdr5p as the main contributor to the PDR response, cell wall signaling via the PKC pathway, and zinc homeostasis, regulated by Zap1p.

Diclofenac, a nonsteroidal anti-inflammatory drug (NSAID), is widely used in the treatment of arthritis and related disorders because of its inhibitory effect on prostaglandin synthesis. Additionally, the antifungal potency of diclofenac has been tested against prostaglandin-secreting pathogenic fungi (3). Unfortunately, diclofenac may cause serious adverse drug reactions (ADRs) that target the liver (25), heart (11), or upper gastrointestinal tract (29). Furthermore, diclofenac is an environmental hazard to Gyps vultures due to its widespread use as a veterinary drug (37). In mammalian hepatocytes, diclofenac toxicity has been linked to mitochondrial dysfunction and oxidative metabolism by cytochrome P450s (15, 30). Gene expression analysis has been performed on murine liver samples (7, 9) and on human and rat hepatocytes (26) treated with diclofenac to further identify the underlying toxicity mechanisms. In particular, genes associated with oxidative stress, cell death, and cell cycle regulation were identified. However, the specific genes directly involved in diclofenac toxicity remained unclear.

Previously, we have shown in *Saccharomyces cerevisiae* that subunits Rip1p and Cox9p of the mitochondrial respiratory chain are diclofenac targets and that metabolism of diclofenac by cytochrome P450s increases its toxicity (43, 44). Yeast is an excellent eukaryotic model organism for toxicological research (20, 45). The advantages of yeast over mammalian cellular systems are its straightforward genetic accessibility, cost-effectiveness, and rapid growth. Furthermore, the pharmacological targets of diclofenac do not exist in yeast, thereby simplifying the test system, whereas many of the mechanisms underlying toxicity and resistance to chemicals and other environmental stresses are conserved (32). For example, both in yeast and in mammalian cells, diclofenac toxicity is related to mitochondrial dysfunction and elevated production of reactive oxygen species (ROS) (15, 30, 43). The availability of a well-annotated genome sequence makes yeast an ideal model system for genome-wide studies. The transcriptional responses of yeast to a wide variety of stress conditions have been studied extensively (6, 12). Moreover, yeast deletion strain collections and overexpression libraries have been used to identify drug targets (14, 31).

In this study, we present the mechanisms of diclofenac toxicity and resistance in yeast as a eukaryotic model organism. By using microarray analysis of adapted yeast cells and by testing the resistance of relevant haploid deletion strains, we gained insight into the main defense mechanisms circumventing diclofenac toxicity.

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▽ Published ahead of print on 1 July 2011.

MATERIALS AND METHODS

Chemicals and stock solutions. Diclofenac was purchased as its sodium salt from Sigma-Aldrich and was dissolved in dimethyl sulfoxide (DMSO; 100 mM). 2′,7′-Dichlorodihydrofluorescein diacetate was obtained from Alexis Biochemi-

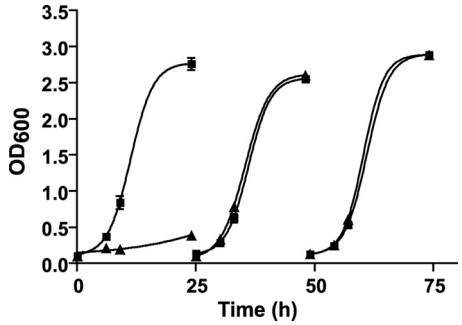

FIG. 1. Yeast cells can adapt to diclofenac. W303 cells were grown in the absence (squares) or presence (triangles) of diclofenac at 100 μM in minimal medium. After 24 and 48 h, the cultures were diluted in minimal medium containing no drug or 100 μM diclofenac. Growth is expressed as the OD_{600} ± SD.

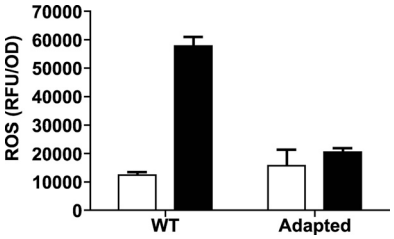

FIG. 2. Diclofenac-adapted cells have lower ROS levels than WT cells in the presence of diclofenac. W303 cells that either were not pretreated (WT) or were pretreated for 48 h with 100 μM diclofenac (adapted) were grown for 3 h with no drug (open bars) or 50 μM diclofenac (filled bars) in the presence of the ROS-sensitive fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate at 10 μM. Data are expressed as fluorescence units corrected for cell density (relative fluorescence units [RFU]) ± SD.

cals and was dissolved in ethyl alcohol (EtOH; 4 mM). Stock solutions were stored at −20°C and were protected from light. All other chemicals were purchased from Sigma-Aldrich at the highest purity.

Strains. The haploid *Saccharomyces cerevisiae* strains W303-1A (*MAT***a** *ura3-52 trp1Δ2 leu2-3,112 his3-11,15 ade2-1 can1-100*) and BY4741 (*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) were used. BY4741 deletion strains were obtained from EUROSCARF. W303-1A Δ*pkc1* and Δ*slt2* strains were a kind gift from Stefan Hohmann (42).

Growth conditions and diclofenac treatment. Strains were grown overnight at 30°C in selective minimal medium (YNB, consisting of 0.67% yeast nitrogen base without amino acids, 2% glucose, and supplemental amino acids and bases). Overnight cultures were diluted in minimal medium and were grown at 30°C to an optical density at 600 nm (OD_{600}) of ~0.2. At this point, diclofenac was added at various concentrations up to 100 μM. Controls were treated with equal amounts of DMSO (maximum, 0.1%). High-performance liquid chromatographic (HPLC) analysis of the medium or cellular lysates revealed no degradation products of diclofenac in wild-type (WT) cells after incubation for 24 h (43).

RNA extraction and microarray analysis. Five independent yeast cultures were incubated with 100 μM diclofenac for 75 h. Every 24 h, cultures were diluted to an OD_{600} of ~0.1 in YNB containing 100 μM diclofenac. Five independent control cultures were grown in the absence of diclofenac. Yeast pellets were frozen at −80°C prior to RNA extraction. mRNA was isolated using the Qiagen RNeasy Mini kit. Hybridization and array analysis were performed by the MicroArray Department of the University of Amsterdam using GeneChip Yeast Genome 2.0 arrays from Affymetrix. Data were normalized using MAS5 and RMA and were analyzed for significantly enriched classes or categories of genes in the Gene Ontology (GO) database using T-profiler (4). T-profiler analysis was also performed to search for significantly enriched groups of genes containing upstream matches to a consensus transcription factor binding motif.

Overexpression constructs. *PDR5* and *SNQ2* overexpression constructs in YEplac181 (origin, 2μm; *LEU2* marker) were a kind gift from Karl Kuchler (33). Scott Moye-Rowley kindly provided the *RSB1* overexpression construct pRS426-*RSB1* (origin, 2μm; *URA3* marker). The plasmids were transformed into yeast strain BY4741 by using the freeze-thaw method (23).

β-Galactosidase reporter assay. The *TRP5*-, *PDR3*-, *PDR5*-, *RSB1*-, and *SNQ2-lacZ* reporter constructs in the low-copy-number vector pSEYC102 were a kind gift from Scott Moye-Rowley (8, 16, 21, 38). The plasmids were transformed into yeast strain BY4741 by using the freeze-thaw method (23). Yeast cultures were grown at 30°C to an OD_{600} of ~0.2. Diclofenac was added, and cultures were incubated for 2 h. Protein extracts were made in LacZ buffer (40 mM Na_2HPO_4, 60 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol) by vortexing with glass beads. Approximately 20 μg of protein was incubated with 24 μg chlorophenol red-β-D-galactopyranoside at 30°C for ~30 min. Absorption at 575 nm was measured and corrected for the incubation time and the concentration of protein in the samples.

Measurement of ROS production. Exponentially growing cultures (2 ml) at an OD_{600} of ~0.2 were either left untreated or treated with 50 μM diclofenac in the presence of the ROS-sensitive fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate at 10 μM. After 3 h at 30°C, the cultures were centrifuged (3 min, 3,000 rpm), and cell pellets were washed and resuspended in 1 ml water. Fluorescence (excitation wavelength [λ_{ex}], 485 nm; emission wavelength [λ_{em}], 535 nm) was measured and corrected for the cell density in the samples.

Figures and statistics. All experiments were performed at least twice in triplicate. Standard deviations (SD) were calculated using GraphPad Prism 4, and figures were created with GraphPad Prism 4 and Adobe Illustrator CS5. The contrast of the photographs in Fig. 6D was enhanced using Adobe Photoshop CS5.

Microarray data accession number. The microarray data determined in this study can be downloaded from the Genome Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo) under accession number GSE29331.

RESULTS

Adaptation of yeast to diclofenac. Previously, we reported that yeast cell growth is inhibited at diclofenac concentrations of 50 μM and higher (43). Upon incubation with 100 μM diclofenac, wild-type yeast cells could hardly grow at all, and after 3 h, only 10% of the cells were viable. However, when cells, after a 24-h treatment with diclofenac, were diluted and treated again with 100 μM diclofenac, no toxicity could be observed any more (Fig. 1), showing that the surviving yeast cells had fully adapted to diclofenac.

Diclofenac targets the mitochondria and induces ROS formation during the initial exposure to diclofenac (43). In the present study, we investigated the amount of ROS formed in adapted cells compared with freshly treated cells. Yeast cells were either adapted by growth for 48 h with 100 μM diclofenac or were grown for 48 h without diclofenac. Exponentially growing cells were then incubated for 3 h with no drug or 50 μM diclofenac in the presence of the ROS-sensitive fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate. Although 50 μM diclofenac caused a growth delay in freshly treated cells, this concentration was not lethal (data not shown). ROS levels in adapted cells that were now incubated without diclofenac were comparable to ROS levels in cells grown continuously in the absence of diclofenac (Fig. 2). Interestingly, ROS formation in adapted cells incubated with 50 μM diclofenac was significantly lower than that in freshly treated cells. As we have observed previously (43, 44), there is a clear correlation between diclofenac-induced growth inhibition and ROS formation.

Since loss of mitochondrial DNA (mtDNA) (*rho*^{0} cells) leads to decreased ROS formation and elevated resistance to diclofenac (43), we investigated whether adapted strains lost their mitochondrial DNA. After several days of incubation with 100 μM diclofenac, cells were plated on plates containing either glucose or glycerol and ethanol as carbon sources. Only

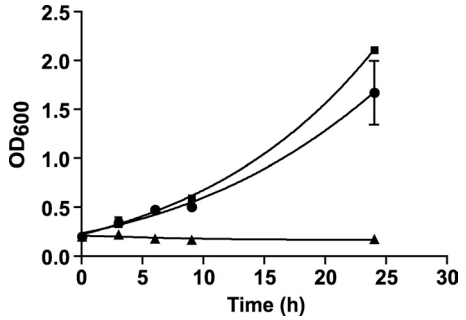

FIG. 3. Adaptation to diclofenac is reversible. W303 strains either were grown in the absence of diclofenac (squares), pretreated for 72 h with 50 μM diclofenac and subsequently incubated with 150 μM diclofenac (circles), or first pretreated for 72 h with 50 μM diclofenac, then incubated for 48 h without diclofenac, and finally incubated with 150 μM diclofenac (triangles). Growth is expressed as the $OD_{600} \pm SD$.

TABLE 2. GO categories of genes whose expression in cells adapted to diclofenac was significantly altered from that in control cells[a]

GO category	Function[b]	T value[c]	E value[c]	Mean
(Drug) transporter activity	F	6.1	1.2E−06	0.38
Endoplasmic reticulum	C	6.0	2.7E−06	0.11
Cell wall	C	4.4	1.4E−02	0.13
Transcription	P	−4.7	4.6E−03	−0.11
Mitochondrion	C	−5.7	1.7E−05	−0.06
rRNA processing	P	−11.4	<1.0E−15	−0.36
Nucleus	C	−11.6	<1.0E−15	−0.12
Ribosome biogenesis	P	−12.5	<1.0E−15	−0.36

[a] Redundant or meaningless GO categories were left out.
[b] F, molecular function; C, cellular component; P, biological process.
[c] T and E values were determined using T-profiler analysis (4) on the whole data set.

cells that are able to respire and thus contain mtDNA can grow on glycerol/ethanol plates. Approximately 95% of the adapted cells contained mtDNA, a finding comparable to that for cells grown without diclofenac. Although *rho*^{0} cells have a higher resistance to diclofenac, no selection for *rho*^{0} cells occurred.

To further investigate the characteristics of adaptation, strains were adapted by exposure to 50 μM diclofenac for 72 h. When these adapted cells were subsequently treated with 150 μM diclofenac, almost no toxicity was observed (Fig. 3). However, when the adapted strains were grown in the absence of diclofenac for 48 h and were then treated with 150 μM diclofenac, the resistance was lost (Fig. 3). This reversibility of adaptation is consistent with the observation that adapted strains do not lose their mtDNA, and it also excludes selection for a random DNA mutation such as that described for multidrug resistance genes (5).

Genome-wide analysis of cells adapted to diclofenac. Because diclofenac adaptation was reversible, it was probably related to altered gene expression. Genes whose expression is significantly altered in adapted cells might play an important role in tolerance to diclofenac. Therefore, we performed microarray analysis of yeast cells adapted to diclofenac and compared the expression profile to that of controls grown without diclofenac. T-profiler analysis (4) was performed on the complete data set to search for significantly enriched groups of genes containing upstream matches to a consensus transcription factor binding motif. The search for motifs revealed upregulation of pleiotropic drug resistance (PDR) genes and genes targeted by Rlm1p, a transcription factor in the protein kinase C (PKC)-mediated mitogen-activated protein kinase (MAPK) pathway, which is responsive to cell wall stress (Table 1). Also, genes containing an Msn2p or -4p (Msn2p/4p) binding motif and responsive to oxidative or general environmental stress were slightly enriched. However, only Msn2p/4p-responsive genes that also contained a PDR-responsive element in their promoters were found to be upregulated. *MSN2* and *MSN4* themselves were both downregulated in the adapted cells (−1.1× and −1.4×, respectively) and probably are not involved in adaptation to diclofenac. Genes containing rRNA processing element (rRPE) or PAC (polymerase A and C box) motifs in their promoters were downregulated. These genes generally encode ribosomal proteins or proteins involved in rRNA and tRNA synthesis and processing and are downregulated in response to a variety of stresses (12). Also, genes containing zinc-responsive promoters (Zap1p binding motif) were downregulated.

T-profiler was also used to analyze the data for significantly enriched classes or categories of genes using the Gene Ontology (GO) database (Table 2). Redundant or meaningless GO categories were left out. Interestingly, mRNA levels of pro-

TABLE 1. Motif groups that were significantly enriched in diclofenac-adapted strains compared with controls

Motif	T value[a]	E value[a]	Genes[b]
PDR	11.3	<1.0E−15	*AZR1*, *RSB1*, *PDR5*, *GRE2*, *RTA1*, *YGR035C*, *ADY2*, *PDR15*, *YPL088W*, *SNQ2*, *ICT1*, *YLR346c*, *YOR268C*, *YOR1*, *YHR140W*, *YKL071W*, *HXT1*, *PDR18*, *TPO1*, *YLL066W-B*, *PRM5*
RLM1	4.5	8.6E−04	*RTA1*, *YGR035C*, *YPL088W*, *YPS3*, *YCR101C*, *YGL258W-A*, *YAL067W-A*, *YNR066C*, *ADH2*, *CSM4*, *YPL067C*, *PRM5*
ZAP1	−3.0	3.7E−02	*YOR387C*, *ADH4*, *VEL1*, *ZPS1*, *YIL169C*, *ZAP1*, *CHA1*, *YLL053C*, *YGK3*, *HPF1*
rRPE	−6.7	2.5E−09	*YOR378C*, *ADH4*, *VEL1*, *CYB5*, *YAP7*, *BFR2*, *YDL063C*, *SUL1*, *GCV2*, *YOL029C*, *BIO2*, *REX4*
PAC	−8.5	<1.0E−15	*CHA1*, *BFR2*, *GIT1*, *YGK3*
Other (upregulated)			*ECM12*, *YJL213W*, *PFS1*, *MET2*, *PRM4*, *YLR012C*, *IST3*, *DAL4*, *YGR153W*, *YOL038C-A*, *RIM15*
Other (downregulated)			*PCL1*, *PDC6*, *YGR079W*, *AQY2*, *SEO1*, *DAK2*, *IRC7*, *FTR1*, *GRE1*, *YNR063W*, *DAL80*, *FET3*, *HLR1*

[a] T and E values were found using T-profiler analysis (4) on the complete data set.
[b] Genes, grouped by motif, that are ≥2 times up- or downregulated in adapted strains compared with controls.

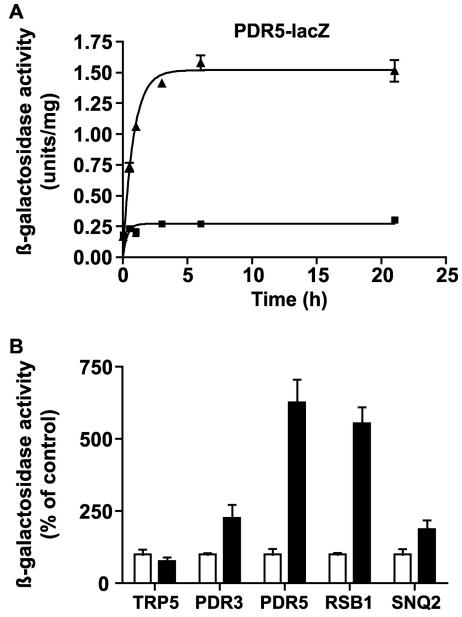

FIG. 4. The pleiotropic drug response (PDR) is dramatically upregulated by diclofenac. (A) Time-dependent expression of *PDR5-lacZ* in a BY4741 strain incubated with no drug (squares) or with 30 μM diclofenac (triangles). Data are expressed as β-galactosidase activity units corrected for the protein concentration ± SD. (B) *TRP5*-, *PDR3*-, *PDR5*-, *RSB1*-, and *SNQ2-lacZ* expression in wild-type BY4741 cells incubated 3 h with no drug (open bars) or with 30 μM diclofenac (filled bars). LacZ expression is presented as a percentage of the β-galactosidase activity for untreated controls (set at100%) ± SD.

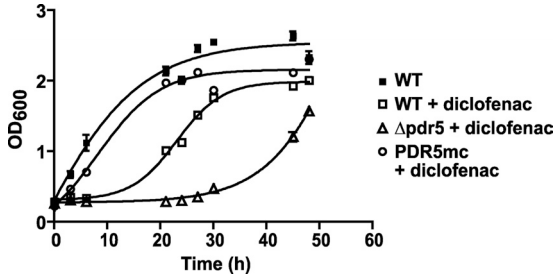

FIG. 5. Pdr5p is important for diclofenac tolerance. BY4741 wild-type (open squares), Δ*pdr5* (open triangles), and PDR5-overexpressing (open circles) cells were grown in the presence of 100 μM diclofenac in minimal medium containing glucose. In the absence of diclofenac, wild-type (filled squares), Δ*pdr5* (not shown), and PDR5-overexpressing (not shown) cells grew comparably to each other. Data are expressed as the OD_{600} ± SD.

teins located in the endoplasmic reticulum (ER) or cell wall were increased, whereas mRNA levels of nuclear and mitochondrial proteins were lowered. Upregulation of the genes encoding cell wall components might be a consequence of *RLM1* upregulation. In agreement with the results obtained by motif analysis, genes involved in drug transport (PDR motif) were upregulated and genes involved in rRNA processing and ribosome biogenesis were downregulated (rRPE and PAC motifs). Downregulation of Zap1p-responsive genes did not lead to significant enrichment of GO category. In summary, the microarray data point to three pathways involved in diclofenac tolerance: the multidrug resistance response, cell wall stress, and zinc homeostasis.

Upregulation of multidrug resistance genes. Our microarray data showed that the pleiotropic drug resistance response is dramatically upregulated in cells adapted to diclofenac. In yeast, multidrug resistance is regulated by transcription factors Pdr1p and Pdr3p (reviewed in reference 36). One of their major targets is the ABC transporter Pdr5p. To investigate the multidrug resistance response during initial diclofenac toxicity, we followed *PDR5* promoter activity using a β-galactosidase reporter construct in cells incubated with 30 μM diclofenac. After the addition of diclofenac, *PDR5-lacZ* expression increased during the first ~3 h, after which it remained stable for at least 21 h (Fig. 4A). *PDR5-lacZ* expression was not changed in cells incubated without diclofenac. Additionally, we measured the promoter activities of the other PDR genes *PDR3*, *RSB1*, and *SNQ2* after a 2-h incubation with 30 μM diclofenac, and we set the level of activity in strains incubated without diclofenac at 100% (Fig. 4B). For these genes also, an increase in *lacZ* expression was observed. The promoter activity of a control gene involved in tryptophan biosynthesis (*TRP5-lacZ*) was not significantly altered. These results show that the PDR response is upregulated during initial diclofenac exposure and remains high in adapted cells.

To investigate the roles of the various PDR transporters in diclofenac resistance, we examined the diclofenac sensitivities of BY4741 strains lacking *AZR1*, *RSB1*, *PDR5*, *ADY2*, *PDR15*, *SNQ2*, *TPO1*, or *PDR12*. *RSB1* and *ADY2* encode long-chain base and acetate transporters, respectively, whereas the other genes encode drug transporters. The wild-type strain BY4741 (Fig. 5) showed diclofenac sensitivity and adaptation similar to those of strain W303 (Fig. 1) and resumed growth after ~15 h with 100 μM diclofenac (Fig. 5). The strain lacking *PDR5* was much more sensitive to diclofenac and needed ~35 h to adapt (Fig. 5). Deletion of any of the other genes tested had no significant effect on diclofenac toxicity (data not shown). Furthermore, we tested the diclofenac sensitivities of strains overexpressing *RSB1*, *PDR5*, or *SNQ2*. Only cells overexpressing *PDR5* were more resistant to diclofenac (Fig. 5); overexpression of the other genes had no effect (data not shown). Additionally, although ~10% of wild-type cells survive a 3-h incubation with 100 μM diclofenac, <2% of the Δ*pdr5* cells and all *PDR5*-overexpressing cells survive these conditions. However, in a plate reader assay, the minimum concentration of diclofenac that completely inhibited growth for 12 h ranged from approximately 75 μM (Δ*pdr5* strain) to 150 μM (WT) and 500 μM (*PDR5*-overexpressing strain) (data not shown). The relatively small differences in these concentrations could indicate that Pdr5p does not actively transport diclofenac but rather indirectly affects diclofenac toxicity via an altered membrane composition (40). Regardless of the exact role of Pdr5p, the growth and survival data at 100 μM diclofenac clearly show the importance of Pdr5p dosage in diclofenac tolerance.

Diclofenac resistance and cell wall modification. T-profiler analysis also showed that Rlm1p-responsive genes were significantly upregulated in diclofenac-adapted cells (Table 1). The transcription factor Rlm1p is phosphorylated by the MAPK Slt2p in the PKC pathway. Therefore, we tested the effect of deleting either *PKC1* or *SLT2* on cell growth in the presence of diclofenac. Both deletion strains showed increased sensitivity

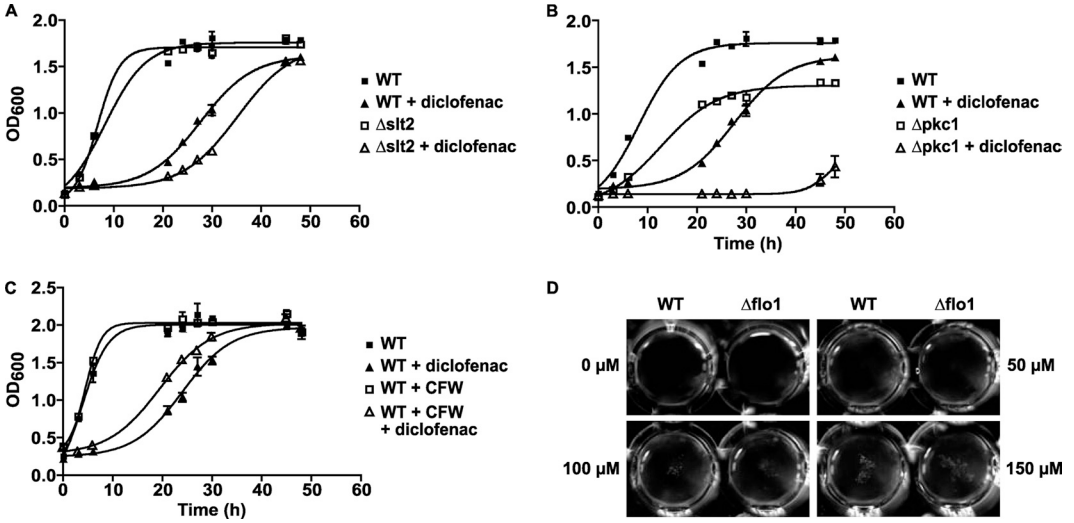

FIG. 6. Diclofenac induces PKC pathway-mediated cell wall stress and flocculation. (A and B) Wild-type (filled symbols), Δ*slt2* (open symbols) (A), and Δ*pkc1* (open symbols) (B) W303 cells were incubated with no drug (squares) or with 100 μM diclofenac (triangles) in minimal medium containing 1 M sorbitol for osmostabilization. (C) Wild-type BY4741 cells were grown in the absence (squares) or presence (triangles) of 100 μM diclofenac in minimal medium containing no additives (filled symbols) or 100 μg/ml calcofluor white (CFW) (open symbols). Growth is expressed as the OD_{600} ± SD. (D) BY4741 WT and Δ*flo1* cells were grown in minimal medium in a 48-well plate. Photographs were taken 20 min after the addition of 50, 100, or 150 μM diclofenac or after addition of DMSO only (0 μM).

to diclofenac (Fig. 6A and B), confirming the important role of the PKC MAPK pathway in diclofenac tolerance.

The PKC pathway is responsive to cell wall integrity, and indeed, many cell wall proteins were upregulated after diclofenac exposure (Table 2). To investigate whether cell wall changes lead to increased diclofenac resistance, cells were incubated with a nontoxic concentration of the cell wall stressor calcofluor white 1 h before the addition of diclofenac. In the presence of calcofluor white, diclofenac toxicity was decreased, whereas the growth of cultures without diclofenac was not affected (Fig. 6C). When calcofluor white was added 1 h after the addition of diclofenac, no decrease in toxicity was observed (data not shown). Similar results were obtained with low concentrations of the cell wall stressor Zymolyase (data not shown). Apparently, the altered cell wall composition induced by calcofluor white decreases diclofenac toxicity.

During the growth assays, we observed that diclofenac caused flocculation (Fig. 6D), which may trigger the cell wall changes. When cells were grown in 48-well plates with continuous shaking, small flocs appeared as early as 10 min after diclofenac addition, suggesting that flocculation is not transcriptionally regulated but rather is a direct effect of diclofenac. Notably, deletion of *FLO1*, encoding a major lectin-like determinant of cell-cell adhesion, did not prevent flocculation (Fig. 6D). Possibly, diclofenac can bind to the cell wall, thereby causing flocculation, cell wall stress, and activation of the PKC MAPK pathway.

Altered zinc homeostasis during diclofenac exposure. The microarray analysis revealed that Zap1p-responsive genes were strongly downregulated (Table 1). Zap1p is a transcription factor that regulates gene expression in response to changes in zinc levels (18). The downregulation of Zap1p and Zap1p-responsive genes suggests that intracellular zinc levels are high in diclofenac-adapted cells. To further explore the role of zinc in diclofenac toxicity, we examined the toxicity of diclofenac in the presence of various zinc concentrations. Addition of 50 to 500 μM $ZnSO_4$ to the medium 1 h before diclofenac addition had no effect on diclofenac toxicity (Fig. 7A [data shown for 50 μm $ZnSO_4$]). However, addition of the zinc chelator 1,10-phenanthroline at 50 μM severely increased diclofenac toxicity (Fig. 7B). The increased toxicity was abolished by the addition of $ZnSO_4$, indicating that the chelation of zinc indeed led to the increased toxicity (Fig. 7B). Since the multidrug resistance response regulators Pdr1p and Pdr3p are zinc transcription factors, the addition of 1,10-phenanthroline might lead to toxicity by preventing the multidrug resistance response. Therefore, we tested the effect of 1,10-phenanthroline on *PDR5-lacZ* expression in the presence of 10 μM diclofenac. This diclofenac concentration is not toxic in combination with 50 μM 1,10-phenanthroline. Both in the absence and in the presence of 1,10-phenanthroline, *PDR5-lacZ* activity was induced ~6 times by 10 μM diclofenac, showing that 1,10-phenanthroline does not interfere with the PDR response (data not shown).

Furthermore, we tested the diclofenac sensitivity of a Δ*zap1* deletion strain. Surprisingly, deletion of Zap1p increased diclofenac toxicity (Fig. 7C). In the presence of 100 μM zinc, the diclofenac resistance of Δ*zap1* cells was increased to the level of wild-type cells, showing that the enhanced diclofenac toxicity of Δ*zap1* strains was caused by a lack of zinc. Accordingly, the addition of 100 μM $FeSO_4$ had no effect on the diclofenac sensitivities of wild-type or Δ*zap1* strains (data not shown). These results point to a pivotal role for zinc in the ability of yeast cells to adapt to diclofenac exposure.

DISCUSSION

In recent decades, many studies have used microarray analysis to examine the response of mammalian cells to a drug of interest (reviewed in reference 47). However, a remaining challenge is to translate these data sets into an actual cellular

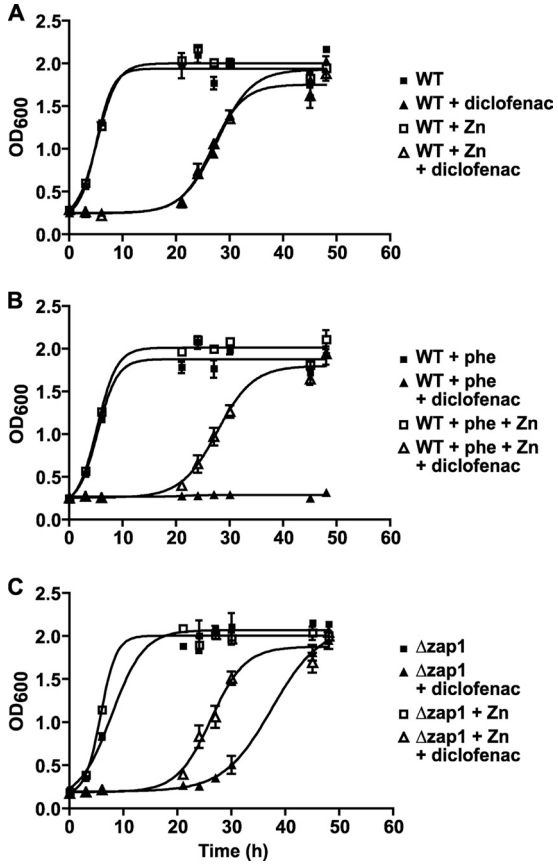

FIG. 7. Diclofenac toxicity is increased under low-zinc conditions. Wild-type (A and B) or Δ*zap1* (C) BY4741 cells were incubated with no drug (squares) or with 100 μM diclofenac (triangles) in minimal medium. (A) The medium contained either no additive (filled symbols) or 50 μM $ZnSO_4$ (open symbols). (B) The medium contained either 50 μM 1,10-phenanthroline (phe) alone (filled symbols) or both 50 μM $ZnSO_4$ and 50 μM 1,10-phenanthroline (open symbols). (C) The medium contained either no additive (filled symbols) or 100 μM $ZnSO_4$ (open symbols). Data are expressed as the OD_{600} ± SD.

target(s). *S. cerevisiae* can be a valuable tool in toxicology studies, since yeast has a well-annotated genome sequence and is easily genetically modified for the validation of potential targets (45). Here we used microarray analysis of diclofenac-adapted yeast cells to identify the cellular pathways involved in diclofenac toxicity or tolerance, and we subsequently used yeast genetics for confirmation of the targets we found.

The major group of upregulated genes in diclofenac-adapted cells consisted of pleiotropic drug resistance genes (Tables 1 and 2). Upregulation of multidrug resistance genes is a common response of yeast to xenobiotics. The resistance of yeast to ~25% of all compounds tested (19, 22) is dependent on the upregulation of these genes. Although several multidrug resistance transporters are upregulated after diclofenac exposure, a single ABC transporter, Pdr5p, is crucial in diclofenac resistance (Fig. 4 and 5). This may be due either to direct transport of diclofenac by Pdr5p or to secondary effects of Pdr5p deletion or overexpression (40). Interestingly, a murine homolog of Pdr5p, BCRP1 (ABCG2), can efficiently transport diclofenac *in vitro* (24). Previously, Mima et al. (34) found that overexpression of the polyamine transporter encoded by *TPO1* decreases sensitivity to diclofenac in yeast. However, deletion of *TPO1* did not alter diclofenac toxicity in our assay. Since Pdr5p is the main transporter involved in diclofenac resistance, the continuous upregulation of *PDR5* is likely to be the main mechanism protecting adapted cells from diclofenac toxicity.

Cells adapted to diclofenac show upregulation of Rlm1p-responsive genes (Table 1). Rlm1p is part of the PKC pathway, which is activated in response to various conditions causing cell wall stress (28). Interestingly, cell wall structural alterations have been observed with many weak acids (pK_a of diclofenac, ~4.0) and possibly reduce the diffusion of the weak acid into the cell (35). Deletion of either *PKC1* or *SLT2* increased diclofenac toxicity (Fig. 6A and B), confirming the role of the PKC pathway in diclofenac resistance. Rlm1p-responsive genes are also upregulated by Zymolyase, which hydrolyzes the β-1,3-glucan network, and by calcofluor white, which binds to cell wall polysaccharides. Indeed, cells preincubated with these agents were more resistant to diclofenac (Fig. 6C). Furthermore, we observed that diclofenac induces *FLO1*-independent flocculation (Fig. 6D), which might trigger the cell wall stress response. Together these results show that diclofenac adaptation involves altered cell wall synthesis and that cell wall alterations protect against diclofenac toxicity.

Although the diclofenac sensitivities of Δ*pdr5* and Δ*pkc1* cells are significantly increased, both strains can adapt to 100 μM diclofenac after incubation for ~35 or ~45 h, respectively (Fig. 5, 6). Interestingly, when these adapted cells were grown without diclofenac for 48 h and were subsequently incubated with 100 μM diclofenac again, their adaptation period was reduced to ~20 h (data not shown). The reduction in adaptation time indicates an acquired mutation, while the remaining 20 h required for adaption reflects the need to make the appropriate gene expression changes that are essential for growth in the presence of diclofenac. Thus, in contrast to our findings for wild-type cells (Fig. 3), adaptation of Δ*pdr5* and Δ*pkc1* cells involves the selection of a mutation. The growth of these mutant Δ*pdr5* cells in the absence of diclofenac was comparable to that of the parental strain, but the mutant Δ*pkc1* cells grew faster without diclofenac than the original Δ*pkc1* cells, providing further evidence of their altered genetic makeup (data not shown). We have excluded selection for loss of mitochondrial DNA in both adapted Δ*pdr5* and Δ*pkc1* cells, but further research is required to identify the nature of the mutation.

Downregulation of the zinc-responsive transcription factor Zap1p in diclofenac-adapted yeast cells indicates that intracellular zinc levels are altered (18). Zinc is essential for the protection of cells against diclofenac, as evidenced by the fact that lowering of zinc levels by deletion of *ZAP1* or by addition of the zinc chelator 1,10-phenanthroline increased diclofenac toxicity, which could be reversed by the addition of extra zinc (Fig. 7). Since diclofenac induces ROS formation (Fig. 2), the antioxidant properties of zinc (reviewed in reference 39) may protect diclofenac-treated cells from further oxidative damage, as has been described for rats (2). Additionally, diclofenac anions in the cytosol may chelate zinc, thereby lowering the cellular free zinc concentration. A similar process has been described for the chelation of iron by lactic acid (1) or for the chelation of both iron and zinc by hop iso-α-acids (17). Interestingly,

lowering of zinc levels by diclofenac has also been observed in patients (41).

Downregulation of genes involved in ribosome biogenesis or other aspects of protein synthesis and cellular growth is a common response to stress and part of the "environmental stress response" (ESR) (6, 12). The typical upregulation of the genes encoding the Msn2p/Msn4p targets in the ESR is not clearly observed here. Interestingly, the downregulation of rRPE and PAC genes has been reported to be transient (10, 12). However, expression of these genes is still altered after 75 h with diclofenac, while no toxicity is observed in terms of the growth rate. Possibly, adapted cells are still stressed, causing downregulation of the rRPE and PAC genes.

Remarkably, adaptation does not mimic typical weak acid adaptation with upregulation of H^+-ATPases and the Msn2p/Msn4p, Haa1p, and War1p regulons (27, 35). Also, although in both yeast (43) and mammalian (15) cells the toxicity of diclofenac is directed primarily toward mitochondria, resulting in increased ROS levels, no oxidative stress response, with upregulated superoxide dismutases, peroxiredoxins, and catalases, is apparent. The lack of a mitochondrial retrograde response by upregulation of *RTG1*, *RTG2*, *RTG3* indicates that the mitochondria are functional; indeed, we could not find selection for *rho*^{0} strains during adaptation to diclofenac. T-profiler analysis, however, shows reduced expression of mitochondrial genes, which might contribute to the reduced sensitivity. Apparently, oxidative stress, and possibly also weak-acid stress, is transient and is involved only in initial diclofenac toxicity. In agreement with this, ROS levels in adapted strains are lower than those in freshly treated strains (Fig. 2). Other cellular changes during adaptation, such as increased Pdr5p expression, may lower diclofenac levels sufficiently to prevent oxidative stress.

In conclusion, we used microarray analysis of diclofenac-adapted strains to identify several processes involved in diclofenac tolerance. By using yeast, we could directly verify the involvement of differentially expressed genes in diclofenac detoxification by applying yeast genetics. Limited transferability of transcriptionally altered genes to genes showing a growth phenotype has been described in many studies (13, 46). By using fully adapted strains for transcriptional analysis, we removed initial lethality responses from the results and linked several significantly enriched motif groups and GO categories to diclofenac toxicity. Especially, upregulation of the multidrug transporter Pdr5p increases resistance to diclofenac. Changes in cell wall composition and zinc homeostasis further contribute to diclofenac tolerance. It remains to be seen whether the importance of zinc for diclofenac tolerance in yeast, discovered here, is relevant for patients with a zinc deficiency (41).

ACKNOWLEDGMENTS

We thank Gertien Smits and Stefan Hohmann for yeast strains, Scott Moye-Rowley and Karl Kuchler for reporter and overexpression constructs, Kevin Augustijn for photographic assistance, and Marco Siderius for useful discussions.

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