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Drug-Induced Endoplasmic Reticulum and Oxidative Stress Responses Independently Sensitize Toward TNFα-Mediated Hepatotoxicity

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Drug-induced liver injury (DILI) is an important clinical problem. Here, we used a genomics approach to in detail investigate the hypothesis that critical drug-induced toxicity pathways act in synergy with the pro-inflammatory cytokine tumor necrosis factor α (TNFα) to cause cell death of liver HepG2 cells. Transcriptomics of the cell injury stress response pathways initiated by two hepatoxicants, diclofenac and carbamazepine, revealed the endoplasmic reticulum (ER) stress/translational initiation signaling and nuclear factor-erythroid 2 (NF-E2)-related factor 2 (Nrf2) antioxidant signaling as two major affected pathways, which was similar to that observed for the majority of ∼80 DILI compounds in primary human hepatocytes. Compounds displaying weak or no TNFα synergism, namely ketoconazole, nefazodone, and methotrexate, failed to synchronously induce both pathways. The ER stress induced was primarily related to protein kinase R-like ER kinase (PERK) and activating transcription factor 4 (ATF4) activation and subsequent expression of C/EBP homologous protein (CHOP), which was all independent of TNFα signaling. Identical ATF4 dependent transcriptional programs were observed in primary human hepatocytes as well as primary precision-cut human liver slices. Targeted RNA interference studies revealed that whereas ER stress signaling through inositol-requiring enzyme 1α (IRE1α) and activating transcription factor 6 (ATF6) acted cytoprotective, activation of the ER stress protein kinase PERK and subsequent expression of CHOP was pivotal for the onset of drug/TNFα-induced apoptosis. Whereas inhibition of the Nrf2-dependent adaptive oxidative stress response enhanced the drug/TNFα cytotoxicity, Nrf2 signaling did not affect CHOP expression. Both hepatotoxic drugs enhanced expression of the translational initiation factor EIF4A1, which was essential for CHOP expression and drug/TNFα-mediated cell killing. Our data support a model in which enhanced drug-induced translation initiates PERK-mediated CHOP signaling in an EIF4A1 dependent manner, thereby sensitizing toward caspase-8-dependent TNFα-induced apoptosis.

Key words: **drug-induced liver injury; transcriptomics; RNA interference; high content microscopy.**

Drug-induced liver injuries (DILIs) constitute an important problem both in the clinic as well as during drug development. The underlying cellular mechanisms that determine the susceptibility toward developing DILI are incompletely understood. Recent data indicate that the crosstalk between drug reactive metabolite-mediated intracellular stress responses and cytokine-mediated pro-apoptotic signaling are important components in the pathophysiology of DILI (Fredriksson *et al.*, 2011; Roth and Ganey, 2011). Tumor necrosis factor α (TNFα) severely enhances liver damage caused by various xenobiotics (Cosgrove *et al.*, 2009; Fredriksson *et al.*, 2011; Lu *et al.*, 2012; Shaw *et al.*, 2007) and it is the major cytokine to be excreted by the liver stationary macrophages (Kupffer cells) upon exposure to bacterial endotoxins such as lipopolysaccharide (LPS) or as a response to hepatocyte damage (Roberts *et al.*, 2007). In addition, reactive drug metabolites covalently modify cellular macromolecules leading to intracellular biochemical perturbations and the induction of various intracellular stress signaling or toxicity pathways, which have been termed the overall human toxome. These toxicity pathways set in motion, and a decreased adaptive response for cell damage recovery and protection, will predispose cells to cell death. Furthermore, it is likely that the onset of diverse sets of stress signaling pathways is causal for the sensitization of the crosstalk with the cytokine signaling. Cosgrove *et al.* previously identified that the Akt, p70 S6 kinase, MEK-ERK, and p38-HSP27 signaling pathways play a role in drug-cytokine synergistic cytotoxicity (Cosgrove *et al.*, 2010). Yet systematic transcriptomics of hepatocytes of both human and rodent origin both *in vitro* and *in vivo* have revealed a diversity of toxicity pathways that are activated by hepatotoxic drugs (Cui and Paules, 2010; Roth and Ganey, 2011). The exact functional contribution of these pathways to DILI has only limitedly

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been studied and so far it remains unclear which drug-induced toxicity pathways modulate the pro-apoptotic activity of TNFα signaling in drug-induced liver cell injury. Here, based on our own transcriptomic analyses, we have focused on the Kelch-like ECH-associated protein 1 (Keap1)/nuclear factor-erythroid 2 (NF-E2)-related factor 2 (Nrf2) antioxidant response pathway and the endoplasmic reticulum (ER) stress-mediated unfolded protein response (UPR).

The Keap1/Nrf2 pathway is important in the recognition of reactive metabolites and/or cellular oxidative stress (Jaiswal, 2004). Under normal conditions, Nrf2 is maintained in the cytoplasm and guided toward proteasomal degradation by Keap1 (Kobayashi *et al.*, 2004). Nucleophilic reactions with the redox-sensitive cysteine residues of Keap1 release Nrf2 followed by its nuclear entry and transcriptional activation of antioxidant genes (Jaiswal, 2004). Nrf2 signaling is critical in the cytoprotective response against reactive metabolites both *in vitro* and *in vivo* (Copple *et al.*, 2008; Okawa *et al.*, 2006), but its role in regulating TNFα pro-apoptotic signaling in relation to DILI is unclear.

The ER stress-mediated UPR is an adaptive stress response to ER protein overload due to enhanced translation and/or perturbed protein folding (Hetz, 2012). It involves expression of molecular chaperones such as the heat shock family member HSPA5 (also known as BiP or Grp78) (Hetz, 2012). When adaptation fails, a pro-apoptotic program to eliminate the injured cell is initiated (Woehlbier and Hetz, 2011). The ER stress response contains three signaling arms: the protein kinase R-like ER kinase (PERK), the activating transcription factor 6 (ATF6), and the inositol-requiring enzyme 1α (IRE1α) (Hetz, 2012). Activation of IRE1α and ATF6 initiates protective responses, whereas activation of PERK leads to attenuation of global protein synthesis and favored translation of activating transcription factor 4 (ATF4) by phosphorylation of eukaryotic initiation factor 2 α (eIF2α), resulting in expression of the ATF4 downstream target gene DDIT3 encoding the C/EBP homologous protein (CHOP) (Harding *et al.*, 2000). CHOP initiates a pro-apoptotic program by modulation of Bcl2-family proteins (Hetz, 2012; Woehlbier and Hetz, 2011). Although ER stress has previously been implicated in DILI (Dara *et al.*, 2011), the role and mechanism of individual ER stress signaling components in controlling DILI in relation to TNFα-induced apoptosis remains undefined.

Here we demonstrate that two different hepatotoxic drugs, diclofenac (DCF) and carbamazepine (CBZ), show a synergistic apoptotic response with the pro-inflammatory cytokine TNFα. Genome-wide transcriptomics revealed an activation of the Nrf2-related oxidative stress response and translation initiation signaling pathway in conjunction with ER stress responses as the most important cell toxicity pathways, which were activated independent of, and preceding, TNFα-mediated cell killing. A systematic short interfering RNA (siRNA) mediated knockdown approach of genes related to these stress-induced pathways allowed a detailed functional evaluation of the mechanism by which oxidative stress, ER stress, and translational regulation are interrelated in the sensitization toward pro-apoptotic TNFα signaling during DILI.

MATERIALS AND METHODS

Reagents and antibodies. Diclofenac sodium (DCF), carbamazepine (CBZ), nefazodone (NFZ), and ketoconazole (KTZ) were obtained from Sigma (Zwijndrecht, the Netherlands). Methotrexate (MTX) was from Acros Organics (Geel, Belgium). Human recombinant TNFα was acquired from R&D Systems (Abingdon, United Kingdom). AnnexinV-Alexa633 was made as previously described (Puigvert *et al.*, 2010). The antibody against caspase-8, cleaved poly ADP-ribose polymerase (PARP), CHOP, and translation initiation factor EIF4A1 were from Cell Signaling (Bioké, Leiden, Netherlands). The antibody against tubulin was from Sigma and the antibody against phosphorylated protein kinase R-like ER kinase (P-PERK; Thr 981) and Nrf2 was from Santa Cruz (Tebu-Bio, Heerhugowaard, The Netherlands).

Liver cells and slices. Human hepatoma HepG2 cells were obtained from American Type Culture Collection (ATCC, Wesel, Germany), cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 25 U/ml penicillin and 25 μg/ml streptomycin, and used for experiments between passage 5 and 20. Primary mouse hepatocytes were isolated from 8 to 10 weeks old male C57BL/6J mice by a modified two-step collagenase perfusion technique (collagenase type IV, Sigma-Aldrich, Zwijndrecht, The Netherlands) and treated as described previously (van Kesteren *et al.*, 2011). The source of human liver tissue and the preparation and incubation of human precision-cut liver slices were described previously (Hadi *et al.*, 2013). In brief, liver slices (diameter 4 mm, thickness 250 μm) were pre-incubated at 37°C for 1 h individually in a well containing 1.3 ml Williams' medium E with glutamax-1 (Gibco, Paisley, UK), supplemented with 25mM D-glucose and 50 μg/ml gentamicin (Gibco, Paisley, UK) (WEGG medium) in a 12-well plate with shaking (90 times/min) under saturated carbogen atmosphere.

Gene expression profiling. For HepG2 cells drug (500μM DCF, 500μM CBZ, 75μM KTZ, 30μM NFZ, and 50μM MTX) or vehicle (dimethyl sulfoxide [DMSO]) exposure was performed for 8 h followed by the addition of 10 ng/ml TNFα or solvent and incubation for another 6 h. For primary mouse hepatocytes, 46 h after isolation, cells were exposed to either 300μM DCF or the solvent DMSO for 24 h. For human liver slices, the slices were treated with 400μM DCF or the solvent DMSO and incubated for 24 h. RNA was isolated using the RNeasy Plus Mini Kit (Qiagen, Venlo, The Netherlands) and RNA integrity and quality was assessed using the Agilent bioanalyser (Agilent Technologies, Palo Alto, CA). The Affymetrix Human Genome U133 plus PM arrays and Affymetrix Mouse Genome 430 2.0

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GeneChip arrays were used for microarray analysis of human and mouse liver cell samples, respectively, and all performed at ServiceXS B.V. (Leiden, The Netherlands). BRB Array Tools software was used to normalize the Affymetrix CEL file data using the Robust Multichip Average (RMA) method. Significantly differentially expressed genes (DEGs) (p-value < 0.001) between the various experimental conditions were identified with an ANOVA test followed by FDR calculation according to Benjamini and Hochberg (Benjamini and Hochberg, 1995). Classification of the selected genes according to their biological and toxicological functions was performed using the Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Redwood, CA). Heatmap representations and hierarchical clustering (using Pearson correlation) were performed using the MultiExperiment Viewer software (Saeed *et al.*, 2003). The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO) (Edgar *et al.*, 2002) and are accessible through GEO Series accession number GSE54257 (http://www.ncbi.nlm.nih.gov/projects/geo/query/acc.cgi?acc=GSE54257).

Gene expression analysis from primary human hepatocytes using the TG-GATEs data set. CEL files were downloaded from the Open TG-GATEs database: "Toxicogenomics Project and Toxicogenomics Informatics Project under CC Attribution-Share Alike 2.1 Japan" http://dbarchive.biosciencedbc.jp/en/open-tggates/desc.html. Probe annotation and probe mapping was performed using the hgu133plus2.db and .cdf packages version 2.9.0 available from the Bioconductor project (http://www.bioconductor.org) for the R statistical language (http://cran.r-project.org). Probe-wise background correction and between-array normalization was performed using the vsn2 algorithm (VSN package version 3.28.0) (Huber *et al.*, 2002). Probe set summaries were calculated with the median polish algorithm of RMA (robust multi-array average) (LIMMA package, version 1.22.0) (Irizarry *et al.*, 2003). The normalized data were statistically analyzed for differential gene expression using a linear model with coefficients for each experimental group (fixed) (Smyth, 2004; Wolfinger *et al.*, 2001). A contrast analysis was applied to compare each exposure with the corresponding vehicle control. For hypothesis testing the moderated t-statistics by empirical Bayes moderation was used followed by an implementation of the multiple testing correction of Benjamini and Hochberg (1995) using the LIMMA package (Smyth, 2004).

RNA interference. Transient knockdowns (72 h) of individual target genes were achieved in HepG2 cells before CBZ/TNFα (500μM/10 ng/ml) and DCF (500μM/10 ng/ml) exposure, using siGENOME SMARTpool siRNA reagents and siGENOME single siRNA sequences (50nM; Dharmacon Thermo Fisher Scientific, Landsmeer, The Netherlands) with INTERFERin siRNA transfection reagent (Polyplus transfection, Leusden, The Netherlands). The negative controls were siGFP or mock transfection. The single siRNA sequences were used to exclude any off-target effects of the SMARTpools resulting in a significant biological effect. The experiments were performed in fourfold and SMARTpool was considered on target when two or more of the four singles showed a similar significant effect. All siRNA-targeted genes can be found in Supplementary table S1.

Cell death assays in HepG2 cells. Induction of apoptosis in real time was quantified using a live cell apoptosis assay essentially the same as previously described (Puigvert *et al.*, 2010).

Western blot analysis. Western blot analysis was essentially performed as previously described (van de Water *et al.*, 1999) using above-mentioned antibodies. Images were processed in Adobe Photoshop CS5 (Adobe, Amsterdam, The Netherlands).

Live cell imaging of GFP-tagged proteins in HepG2 cells. Reporter HepG2 cells for Nrf2 activity (Srxn1 [mouse]) and ER stress (ATF4 and CHOP/DDIT3 [human]) were generated by bacterial artificial chromosome (BAC) recombineering (Hendriks *et al.*, 2012; Poser *et al.*, 2008). Upon validation of correct C-terminal integration of the GFP-cassette by polymerase chain reaction, the BAC-GFP constructs were transfected using Lipofectamine 2000 (Invitrogen, Breda, The Netherlands). Stable HepG2 BAC-GFP reporters were obtained by 500 μg/ml G418 selection. Prior to imaging, nuclei were stained with 100 ng/ml $Hoechst_{33342}$ in complete DMEM. The induction of Srxn1-GFP, ATF4-GFP, and CHOP-GFP expression was followed for a period of 24 h by automated confocal imaging (Nikon TiE2000, Nikon, Amstelveen, The Netherlands). Quantification of the GFP intensity in individual cells was performed using Image Pro Plus (Media Cybernetics, Rockville, USA).

Statistical analysis. All numerical results are expressed as the mean ± standard error of the mean (SEM) and represent data from three independent experiments. Calculations were made using GraphPad Prism 5.00 (GraphPad software, La Jolla). Significance levels were calculated using two-way ANOVA, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

RESULTS

Hepatotoxic Drug Synergy with TNFα is Preceded by Oxidative Stress, ER Stress, and Death Receptor Signaling Gene Expression Networks

First we treated HepG2 cells for 8 h with different compounds associated with unpredictable idiosyncratic DILI in humans, carbamazepine (CBZ), diclofenac (DCF), ketoconazole (KTZ), nefazodone (NFZ), and methotrexate (MTX), at concentrations around $100C_{max}$ for each drug (O'Brien *et al.*, 2006; Xu *et al.*, 2008), followed by an additional incubation with or without TNFα (10 ng/ml) for 16 h. CBZ, DCF, and KTZ showed a significant enhanced apoptosis when combined with TNFα (Fig. 1A). A slight trend toward synergy was observed for NFZ,

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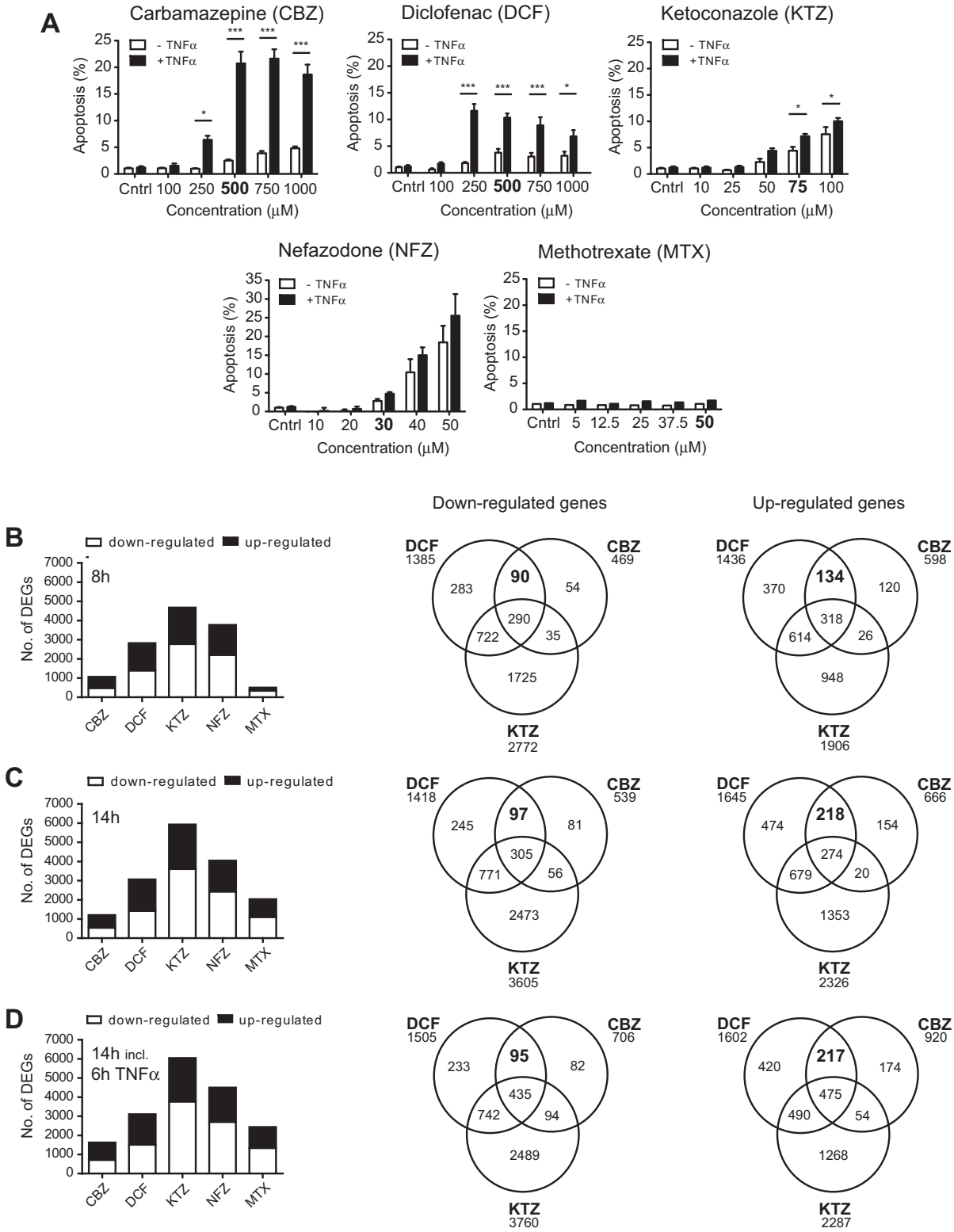

FIG. 1. Apoptosis and gene expression profiling of hepatotoxic drugs and TNFα in HepG2 cells. (A) The apoptosis after drug exposure was followed in real time from 8 to 24 h using automated imaging and AnnexinV (AnxV)-Alexa633 binding to apoptotic cells. The end-points (24 h) are presented as relative AnxV-Alexa633 intensities and the concentrations used for gene expression analysis are marked in bold. The data shown are means of three independent experiments ± SEM. ***$p < 0.001$, *$p < 0.05$. The gene expression after 8 (B), 14 (C), and 14 h including 6 h of TNFα (10 ng/ml; (D)) exposure to diclofenac (DCF), carbamazepine (CBZ), ketoconazole (KTZ), nefazodone (NFZ), and methotrexate (MTX) is presented as number of genes differentially up- (black) or down-regulated (white) compared with control. The total number of genes overlapping among the TNFα-synergizing drugs is shown in the corresponding Venn-diagrams with the overlap between DCF and CBZ in bold.

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whereas hardly any toxicity was observed for MTX with or without TNFα (Fig. 1A).

To find the mechanism behind TNFα synergy we next performed a gene expression analysis on HepG2 cells exposed to DCF (500μM), CBZ (500μM), KTZ (75μM), NFZ (30μM), and MTX (50μM) for 8 h to investigate which intracellular signaling pathways were perturbed by the drugs prior to TNFα addition (Fig. 1B). The concentrations were chosen based on minimal drug-induced toxicity with, if any, maximal TNFα apoptotic synergism (Fig. 1A, in bold). Whereas MTX only mildly affected the gene expression (503 DEGs at 8 h), which was related to the very limited cytotoxicity (Fig. 1A), KTZ caused the strongest gene expression changes (4678 DEGs at 8 h; Fig. 2A) in association with greater onset of cell death (Fig. 1A). Not many additional changes in DEGs were observed after treatment for an additional 6 h, a time-point where synergism with TNFα is apparent (data not shown), with the compounds either in presence or absence of TNFα (Figs. 1C and D). To identify likely candidate genes that contribute to this synergy, we determined the overlap in DEGs for all synergizing drugs (DCF, CBZ, and KTZ; see Venn-diagrams in Figs. 1B–D). Because the most significant TNFα synergy was observed for CBZ (Fig. 1A), we considered this a relevant model compound for further comparisons. DCF showed the highest overlap with CBZ in DEGs when taking into account that the direction of regulation (up or down) should be the same between the two compounds (Figs. 1B–D), and therefore these two drugs were chosen for further detailed analysis.

Next we employed IPA software to identify the toxicity-related signaling pathways that were affected by both CBZ and DCF as early as 8 h after treatment (Fig. 2A). Three prominent toxicity pathways were found: "EIF2-signaling/endoplasmic reticulum stress pathway," "NRF2-mediated oxidative stress response," and "apoptosis/death receptor signaling." We have previously reported the involvement of death receptor signaling under DCF/TNFα conditions (Fredriksson *et al.*, 2011) and we have observed the same effect for CBZ/TNFα (data partially reported in Supplementary table S1).

Subsequently we obtained all the individual genes from IPA that determine the significant pathways described in Figure 2A (Supplementary table S2). Unsupervised hierarchical clustering of all these selected genes allowed identification of three main gene clusters that were up-regulated after 8 h CBZ and/or DCF but not MTX treatment (Supplementary fig. S1A). Interestingly, these contained almost exclusively genes representing the three prominent toxicity pathways (compare Fig. 2A and Supplementary fig. S1A). For further gene selection we used a threshold of 1.5-fold change for any CBZ or DCF treatment time-point (Fig. 2B).

We wanted to ensure that the genes that were significantly regulated in HepG2 cells as presented in Figure 2B after CBZ and DCF exposure were also significantly regulated in primary human hepatocytes after exposure to ~80 drugs, including a large diversity of hepatotoxicants (Chen *et al.*, 2011). In Figure 3, the expression of these genes after drug exposure in the primary human hepatocytes is presented. We also included classical downstream target genes known to be essential in the ER-stress and oxidative stress: XBP1, CHOP/DDIT3, BiP/HSPA5, and SRXN1. The target genes reflecting EIF2-signaling/ER stress/UPR pathway and Nrf2-mediated oxidative stress pathway were mostly affected by the DILI compounds, unlike the death signaling genes, with Bim being the exception. Importantly, CBZ, DCF, as well as KTZ strongly affected the set of these 34 genes in primary human hepatocytes (see Fig. 3 and Supplementary fig. S1B). Moreover, unsupervised clustering of altered expression levels of these 34 genes for all DILI compounds revealed a single cluster with our synergizing compounds with an important addition of sulindac that has previously been reported to synergize with TNFα in another *in vitro* model of idiosyncratic liver injury (Zou *et al.*, 2009). Thus, we confirmed the regulation by CBZ and DCF of a large proportion of the target genes that are central in the EIF2-signaling/ER UPR response and Nrf2-mediated oxidative stress response in primary human hepatocytes.

Oxidative Stress Sensitizes to Diclofenac- and Carbamazepine-Mediated Apoptosis

Nrf2-mediated oxidative stress response was significantly affected by CBZ and DCF (see Fig. 2). Stabilization of Nrf2 after oxidative stress allows its nuclear translocation and transcriptional activation of antioxidant genes (Jaiswal, 2004). DCF caused a stabilization of Nrf2 in HepG2 cells (Fig. 4A), which was less clear for CBZ; TNFα addition did not affect the stabilization of Nrf2 (Fig. 4A). Sulfiredoxin (Srxn1) is a direct target of Nrf2 (Soriano *et al.*, 2009) and we monitored the activity of Nrf2 using live cell imaging of a BAC-Srnx1-GFP HepG2 reporter cell line. In line with the microarray data and the Nrf2 stabilization, Srxn1-GFP expression was strongly induced following both DCF and CBZ treatment (Fig. 4B). We validated that the siRNA-mediated knockdown of Nrf2 (Supplementary fig. S2A) completely inhibited the Srnx1-GFP expression, whereas Keap1 knockdown enhanced the Srxn1-GFP response, supporting the functionality of the Keap1/Nrf2 pathway in these cells (Supplementary fig. S2B). The Nrf2 pathway was also critically involved in the protection against drug/TNFα-mediated cell killing. Knockdown of Keap1 led to enhanced protein levels of Nrf2 (Supplementary fig. S2A), which was associated with a protection against CBZ/TNFα- and DCF/TNFα-induced cytotoxicity and inhibition of caspase-8 activation (Figs. 4C and D and Supplementary fig. S2C). Importantly, knockdown of Nrf2 itself led to enhancement of the apoptosis (Fig. 4C and Supplementary fig. S2C).

These data collectively illustrate the importance of oxidative stress in CBZ/TNFα- and DCF/TNFα-induced cytotoxicity.

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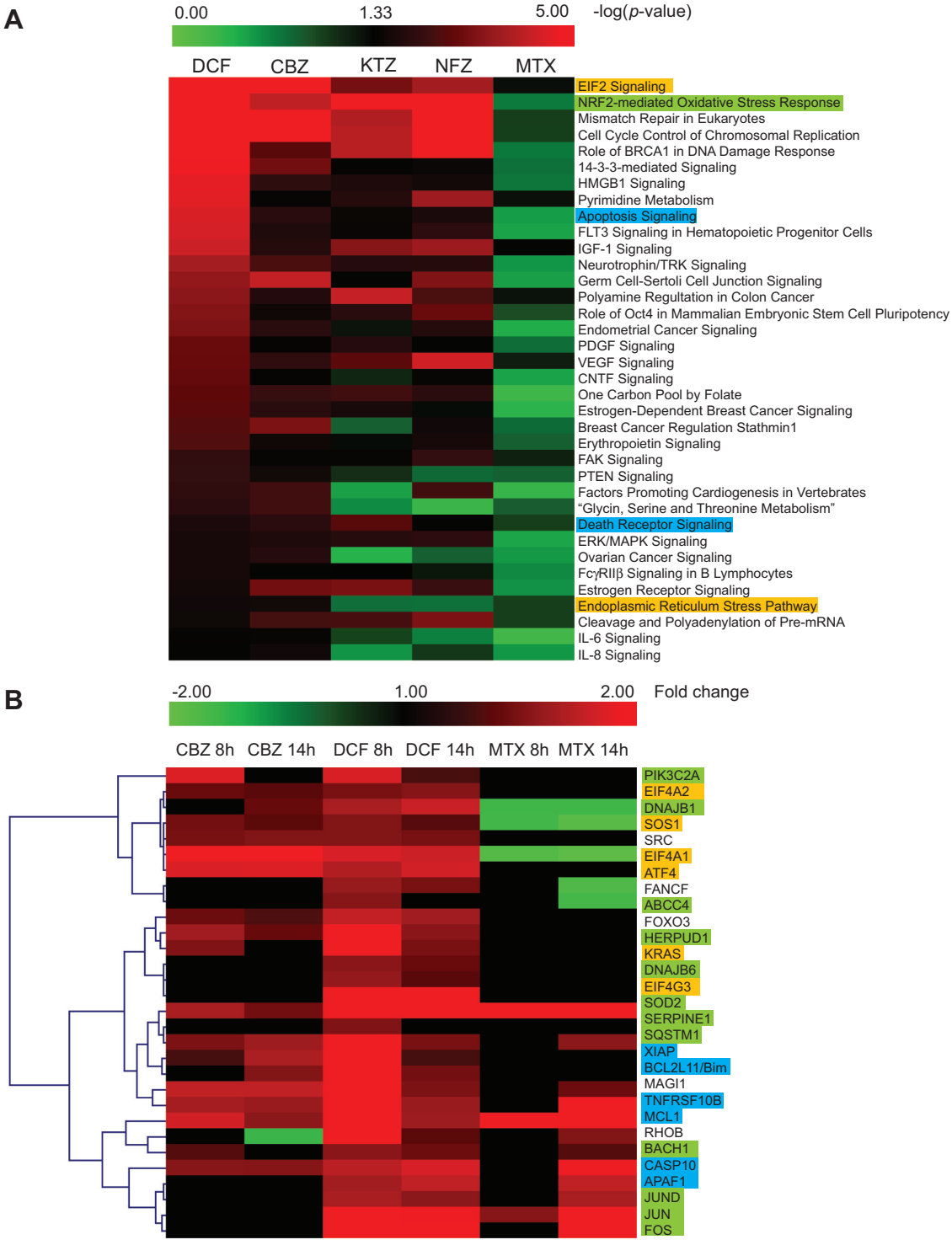

FIG. 2. Identification of CBZ and DCF specific stress responses. (A) Using IPA software the canonical pathways being significantly affected following exposure to diclofenac (DCF; 500μM), carbamazepine (CBZ; 500μM), ketoconazole (KTZ; 75μM), nefazodone (NFZ; 30μM), and methotrexate (MTX; 50μM) for 8 h were determined. The pathways are ranked by the criteria of being significantly regulated after DCF and CBZ, but not after MTX treatment. The most prominent toxicity pathways are highlighted as follows: EIF2 Signaling/Endoplasmic Reticulum Stress Pathways in yellow, Nrf2-mediated Oxidative Stress Response in green and Apoptosis/Death Receptor Signaling in blue. (B) After hierarchical clustering using Pearson correlation and average linkage of the genes representing the pathways in (A), the three clusters showing most genes up-regulated under DCF and CBZ conditions but not MTX after 8 and 14 h exposure are shown and further clustered using the same method. The colors indicate which pathways they belong to according to the highlighting in (A). (For interpretation of the references to color in this figure legend, the reader is referred to the online version of this article.)

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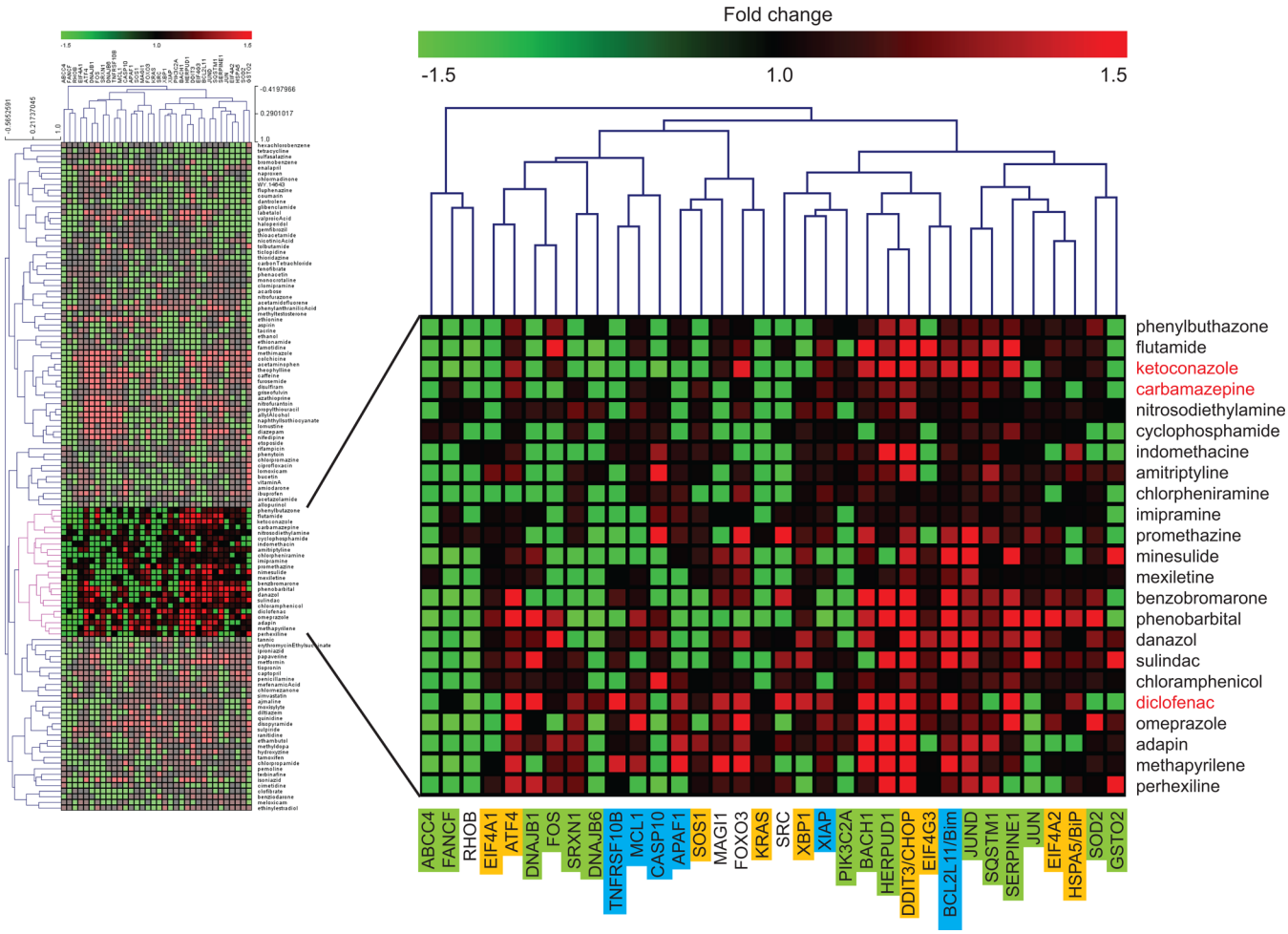

FIG. 3. Induction of carbamazepine and diclofenac specific stress responses by ~80 DILI compounds in primary human hepatocytes. The expression of the genes presented in Figure 2B with an addition of typical endoplasmic reticulum (ER) stress related as well as oxidative stress related genes was investigated in the genes set from TG-GATEs. Hierarchical clustering using Pearson correlation and average linkage resulted in a cluster containing drugs synergizing with TNFα (red). (For interpretation of the references to color in this figure legend, the reader is referred to the online version of this article.)

PERK Activation Determines ER Stress-Mediated Hepatotoxicant/TNFα Synergistic Cell Death

Next we explored the role of the ER stress/UPR pathway in the apoptosis induction after CBZ/TNFα and DCF/TNFα exposure. Pre-treatment of the cells with an ER stressor, tunicamycin, to induce a protective adaptive ER stress response, protected against CBZ/TNFα and DCF/TNFα cell death (Fig. 5A and Supplementary fig. S3A, respectively). Thereafter we systematically analyzed the role of critical upstream signaling components of the ER stress/UPR, IRE1α, ATF6, and PERK (Hetz, 2012). Knockdown of IRE1α and ATF6 led to an enhanced apoptotic response following CBZ/TNFα (Fig. 5B) and DCF/TNFα (Supplementary fig. S3B) exposure whereas knockdown of PERK had a protective effect (Fig. 5B [CBZ] and Supplementary fig. S3B [DCF]). This indicates an exclusive role of the PERK-induced signaling pathway in the onset of apoptosis. In contrast, IRE1α and ATF6 both have a protective role, most likely related to the control of the cytoprotective heat shock protein family member BiP/HSPA5 (Hetz, 2012). Importantly, activation of PERK could also be observed, which started as soon as 2 h after exposure to both CBZ and DCF, independent of TNFα (Fig. 5C).

Next we determined the overall activation of the different ER stress programs after drug exposure, and focused on the PERK/ATF4, IRE1α/XBP1, and ATF6 pathway activities. For this we evaluated the differential expression of downstream target genes of the transcription factors ATF4, XBP1, and ATF6 after CBZ and DCF exposure. ATF4 showed the strongest upregulation of downstream targets supporting the hypothesis of a more important role for PERK/ATF4 signaling in the drug-induced toxicity compared with ATF6 and IRE1α/XBP1 (Fig. 6A). To certify that this main activation of ATF4 after drug exposure was not selective for HepG2 cells, we determined for CBZ and DCF the differential expression of UPR target genes

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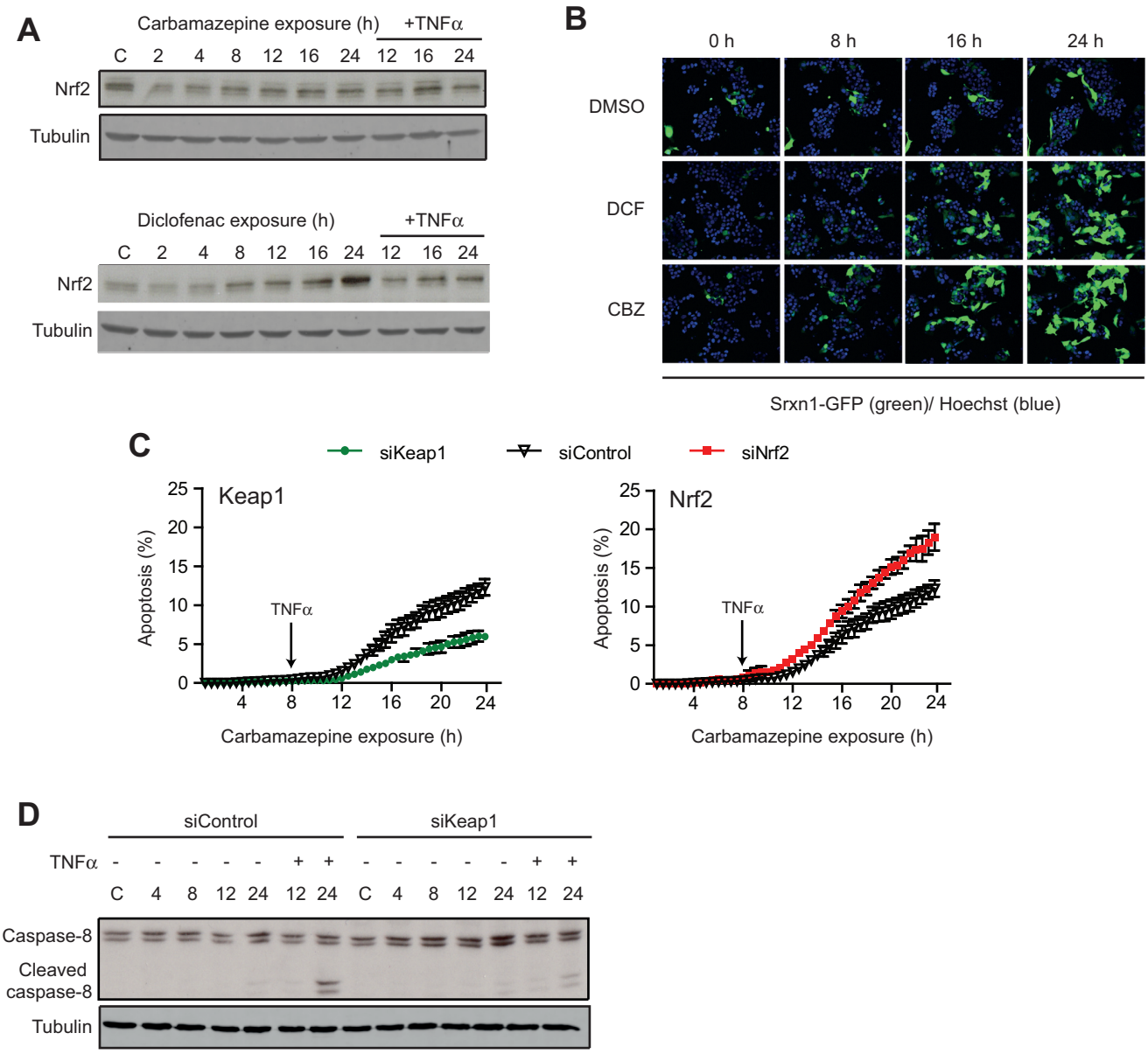

FIG. 4. Carbamazepine and diclofenac induce an Nrf2-response affecting the drug/TNFα-induced apoptosis. (A) Nrf2 protein levels were investigated by Western blot analysis following carbamazepine (CBZ; 500μM) and diclofenac (DCF; 500μM) exposure ± TNFα addition. "C," controls exposed to vehicle (DMSO) for 12 h. (B) Nrf2-responsive Srxn1-GFP levels were followed using automated confocal microscopy. Shown are representative images of Srxn1-GFP (green) HepG2 cells exposed to DMSO, DCF, or CBZ for 0, 8, 16, and 24 h. Nuclei are stained by Hoechst (blue). (C) The effect on CBZ/TNFα (10 ng/ml) induced apoptosis after Nrf2 and Keap1 knockdown (SMARTpool) was investigated using live cell imaging of apoptosis. (D) Apoptosis induced via the extrinsic pathway, as measured by protein expression of cleaved caspase-8 using Western blot, was investigated after Keap1 knockdown and a time series of CBZ exposure ± TNFα addition. Tubulin serves as loading control and "C" is control exposed to vehicle for 12 h. The data are presented as means of three independent experiments ± SEM or representative of three independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the online version of this article.)

under control of ATF4, XBP1, and ATF6 also in the three different primary hepatocyte models: precision-cut human liver slices (HLS), primary human hepatocytes (PHM), and primary mouse hepatocytes (PMH). Importantly, also in these primary cell systems, the ATF4 transcriptional activity appeared superior to the one of ATF6 and XBP1 after exposure to CBZ or DCF (Fig. 6B, for gene labels see Supplementary fig. S4).

Finally, we determined whether the PERK/ATF4 pathway was functional in the HepG2 cells. For this we generated a HepG2 BAC-ATF4-GFP reporter cell line and applied automated live cell confocal microscopy to determine the up-

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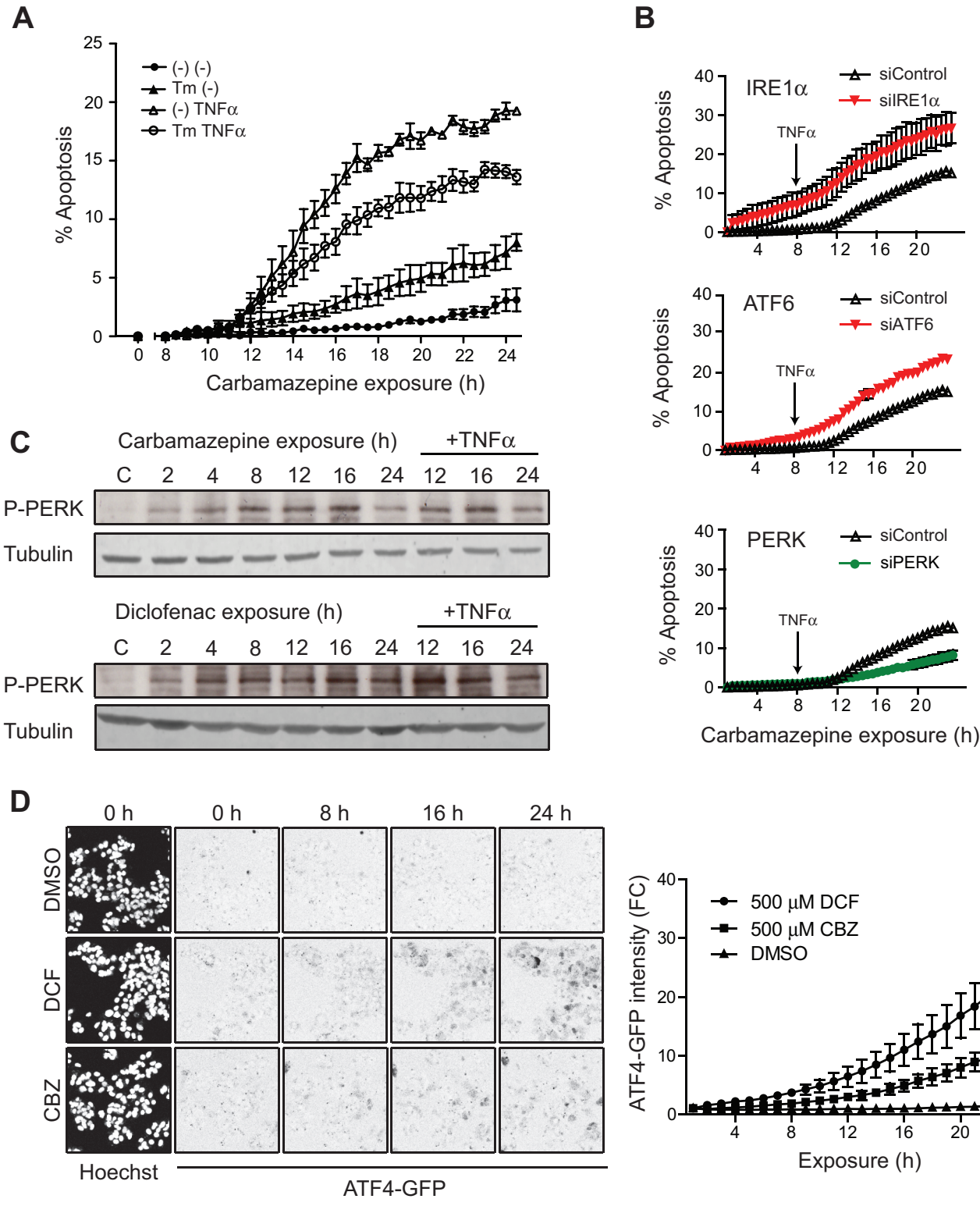

FIG. 5. Carbamazepine and diclofenac induce an ER stress response affecting the drug/TNFα-induced apoptosis. (A) HepG2 cells were pre-treated with ER-stressor tunicamycin (Tm; 10 μg/ml; (A)) for 16 h before treatment with 500μM carbamazepine (CBZ). TNFα (10 ng/ml) was added 8 h after drug exposure. (B) Apoptosis induced by CBZ/TNFα after knockdown (SMARTpool) of the UPR mediators IRE-1α, ATF6, and PERK was followed in time by automated imaging of AnxV-Alexa633 staining. (C) PERK activation following CBZ and diclofenac (DCF; 500μM) exposure was followed in time by Western blotting for phosphorylated PERK (P-PERK). "C," control exposed to vehicle for 12 h. (D) HepG2 cells expressing BAC-ATF4-GFP were followed in time after exposure to DCF, CBZ (500μM), or vehicle (DMSO) using automated confocal microscopy. Representative images of Hoechst at 0 and ATF4-GFP (inverted) at 0, 8, 16, and 24 h after drug exposure are shown as well as the quantification of the increase in ATF4-GFP intensity in time after DCF and CBZ exposure. Values are presented as fold changes (FC) of time-point 0 and the data are presented as means of three independent experiments ± SEM.

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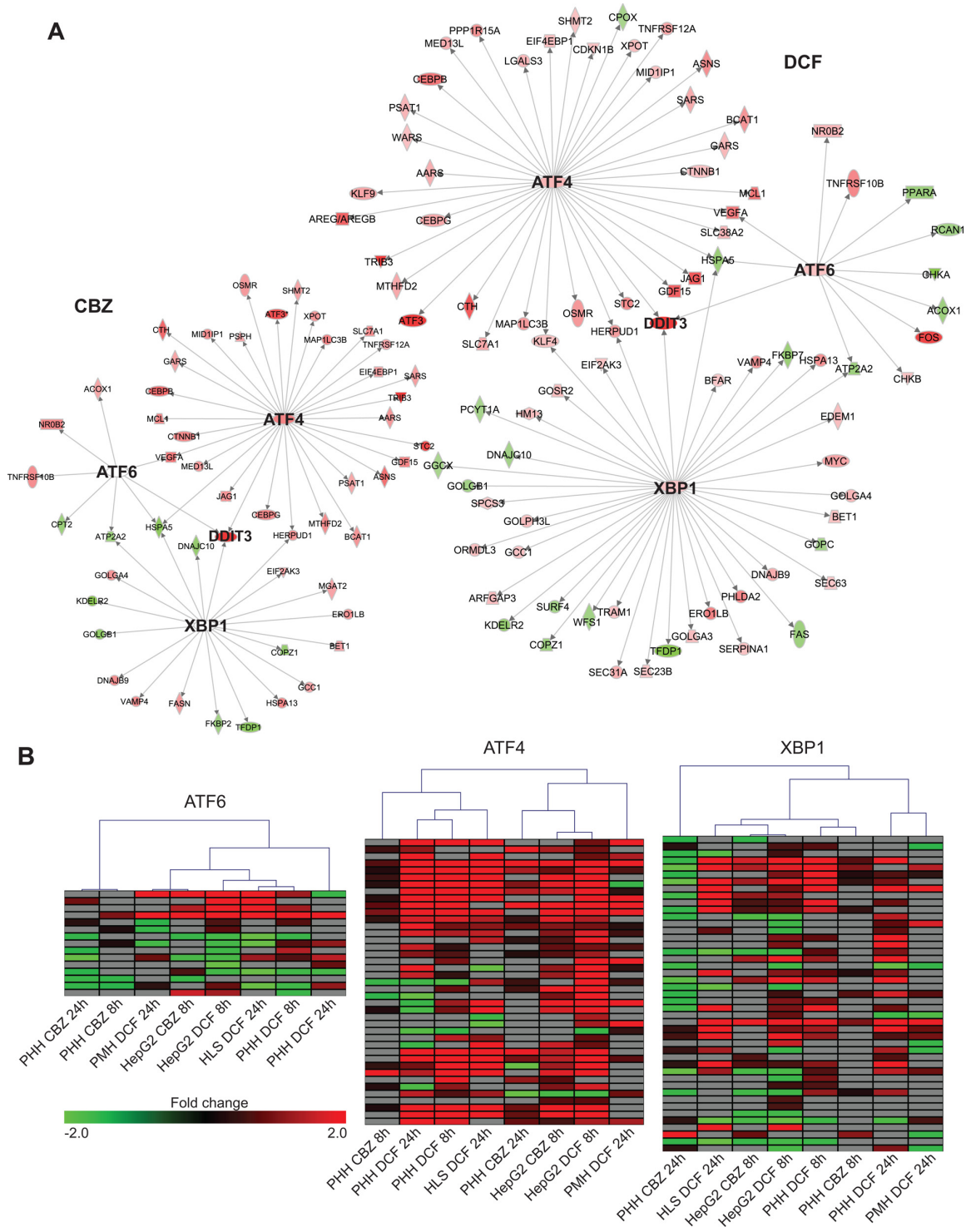

FIG. 6. Diclofenac and carbamazepine exposure mainly induces ATF4 transcription. (A) Using the IPA software the genes up- or down-regulated by the transcription factors downstream of the three distinct unfolded protein response pathways, PERK (ATF4), IRE-1α (XBP1), and ATF6 were determined after 8 h carbamazepine (CBZ) or diclofenac (DCF) exposure. Red coloring of the shapes indicates up-regulation of the target genes, whereas green indicates down-regulation. The intensity reflects the fold-change gene expression compared with vehicle-exposed cells. (B) Expression of the genes induced by DCF and CBZ in HepG2 cells was determined by gene array analysis of primary human (PHH), human liver slices (HLS), and primary mouse hepatocytes (PMH) at 8 and/or 24 h of CBZ and DCF exposure followed by hierarchical clustering of the *in vitro* systems using Pearson correlation and average linkage. Gray color indicates genes that are not regulated at this significance level. A complete figure containing gene names is represented in Supplementary figure S4. (For interpretation of the references to color in this figure legend, the reader is referred to the online version of this article.)

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regulation and nuclear translocation of ATF4. ATF4 expression was induced by both DCF and CBZ in time and primarily localized in the nuclear compartment (Fig. 5D) supporting the activation of PERK-mediated UPR signaling pathway and ability to modulate ATF4 target genes.

Carbamazepine and Diclofenac Induce Expression of Pro-Apoptotic CHOP

A major target of PERK-mediated ATF4 activation is the pro-apoptotic transcription factor CHOP/DDIT3 (Harding *et al.*, 2000). We observed a DCF- and CBZ-induced activation of ATF4 (Figs. 5D and 6) and subsequent increased expression of CHOP/DDIT3 to both compounds in both HepG2 cells (Fig. 6A) and primary cell systems including liver slices (Supplementary fig. S4). We established a HepG2 BAC-GFP-CHOP reporter cell line and used automated live cell confocal microscopy to monitor the induction of GFP-CHOP. Whereas GFP-CHOP was absent under control conditions, both CBZ and DCF induced the expression of GFP-CHOP in time (Fig. 7A), which was reproduced in parental HepG2 cells by Western blotting (Fig. 7B). This CHOP induction was critical for the onset of cell death because siRNA-mediated knockdown of CHOP protected against the apoptosis induced by CBZ/TNFα and DCF/TNFα (Fig. 7C and Supplementary fig. S3C). These data strongly support the role for the PERK/ATF4/CHOP program in the cell death induced by DCF/TNFα and CBZ/TNFα.

Carbamazepine-Induced Nrf2 Activation is Independent of ER Stress

Because ER stress can activate the Nrf2 pathway (Cullinan and Diehl, 2006) we wanted to determine the link between ER stress and oxidative stress. Whereas PERK and CHOP knockdown protected against cell death (Figs. 5C and 7C), neither PERK nor CHOP knockdown inhibited the expression of the Nrf2 target gene Srxn1 in the Srxn1-GFP HepG2 reporter cells (Fig. 8A). Vice versa, knockdown of Keap1, which stabilized Nrf2, in association with strong Srxn1 expression and cytoprotection against CBZ/TNFα and DCF/TNFα (see above), did not block the activation of PERK and the expression of CHOP after CBZ exposure (Fig. 8B). Although both important for the DCF/TNFα- and CBZ/TNFα-induced cell injury, the role of the oxidative stress response appears to be unrelated to the PERK-initiated ER-stress response.

EIF4A1 Controls CHOP Expression and Thereby Apoptosis Onset

Eukaryotic initiation factor 2 (EIF2) signaling in relation to translation initiation was the major pathway affected by CBZ and DCF (see Fig. 2). Salubrinal, an inhibitor of the dephosphorylation of translation initiation factor eIF2α, which is protective against ER stress-induced toxicity (Boyce *et al.*, 2005), inhibited the CBZ and DCF synergy with TNFα (Fig. 9A and Supplementary fig. S3D) supporting a central role for the translational program in the onset of drug/TNFα-induced apoptosis. To further test this hypothesis we performed a knockdown of the RNA helicase EIF4A1, the translation initiation factor that was found most up-regulated after DCF and CBZ exposure (Fig. 2B). Depletion of EIF4A1 provided an almost complete protection against both CBZ/TNFα- and DCF/TNFα-induced apoptosis (Fig. 9B and Supplementary fig. S3E).

Inhibition of global translation is one of the responses for the cell to try to cope with enhanced ER stress (Hetz, 2012). Intriguingly, siEIF4A1 did not affect the PERK activation following CBZ exposure, yet it almost completely inhibited the induction of CHOP (Fig. 9C). Together these data are indicative for a crucial role for translation in the induction of drug/TNFα-induced apoptosis, which is for a major part related to EIF4A1-mediated translation of ER-stress protein CHOP.

DISCUSSION

Here we studied in detail the underlying molecular mechanisms of the synergistic apoptotic response between hepatotoxic drugs and the pro-inflammatory cytokine TNFα using a unique integration of transcriptomics and RNA interference-based functional genomics. Gene expression analysis of HepG2 cells and primary human and mouse hepatocytes as well as human precision-cut liver slices demonstrated the specific activation of the ER-stress/UPR signaling route through the ATF4 transcriptional activity by DCF and CBZ. Further functional analysis of the role of critical determinants of this pathway identified PERK and CHOP as pivotal players in the onset of drug/TNFα-mediated cytotoxicity in HepG2 cells. Importantly, although oxidative stress modulated the onset of cell death, it did not affect the ER-stress/UPR program. On the contrary, the translational machinery, of which translation initiation factor EIF4A1 is a critical marker, was manifested as a major determinant of CHOP expression and, thereby, onset of drug/TNFα-mediated toxicity.

Our data demonstrate a clear enhancement of apoptosis with the addition of TNFα to DCF and CBZ treated HepG2 cells, whereby we chose to focus on the toxicity pathways induced by these two compounds. In contrast, in cells pre-exposed to other known idiosyncratic hepatotoxicants, the synergism with TNFα was not so clear in the case of KTZ, and absent in NFZ and MTX pre-exposed cells. A synergy in the regulation of the expression of genes directly involved in the apoptosis seems unlikely because all five compounds synergized in the expression of various candidate genes (Supplementary fig. S5), although we cannot exclude the role of some individual genes in the DCF/TNF and CBZ/TNF synergistic cytotoxicity. Instead, the discrepancy between synergizing and non-synergizing drugs may be explained by the fact that DCF-induced liver injury has been linked to the involvement of an activated immune system (Deng *et al.*, 2006), and the idiosyncratic nature of CBZ-induced liver injury has been linked to hypersensitivity reactions (Leeder, 1998). However, KTZ, NFZ, and MTX, although reported in-

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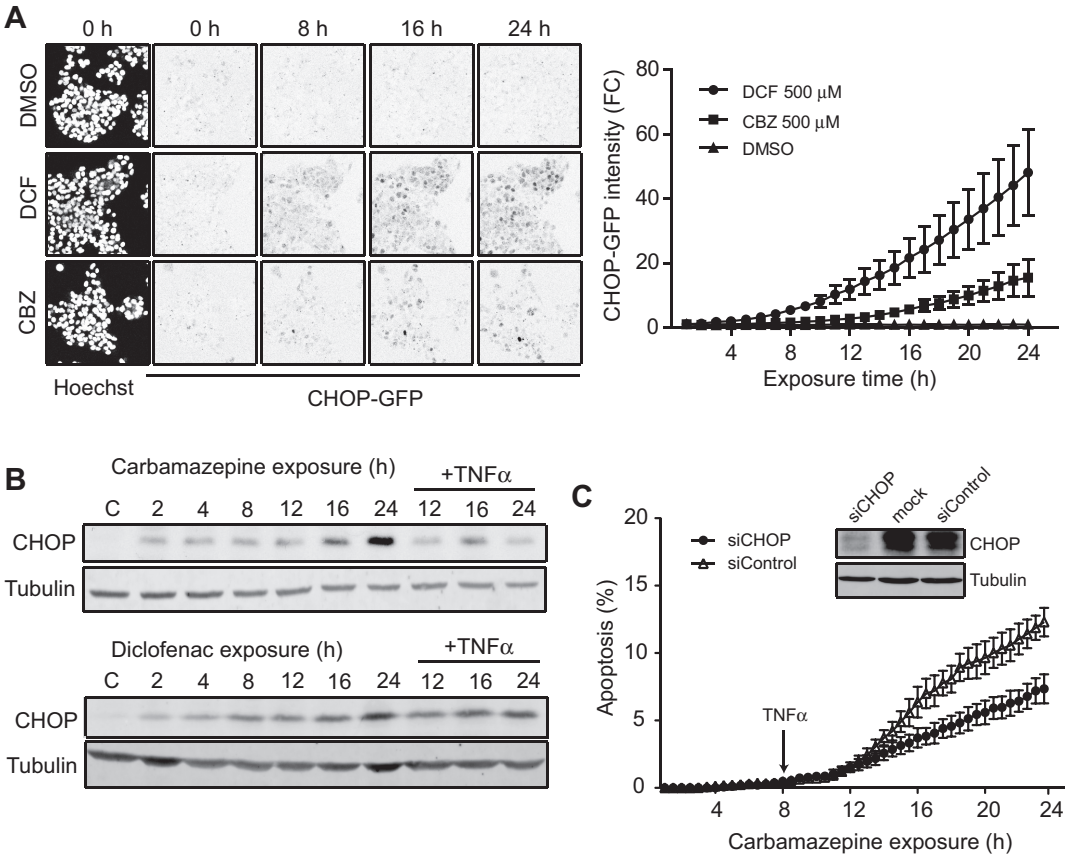

FIG. 7. Carbamazepine- and diclofenac-induced CHOP expression is critical for the apoptosis induction. (A) HepG2 cells expressing BAC-CHOP-GFP were followed in time after exposure to diclofenac (DCF; 500μM), carbamazepine (CBZ; 500μM), or vehicle (DMSO) using automated confocal microscopy. Representative images of Hoechst at 0 and CHOP-GFP at 0, 8, 16, and 24 h after drug exposure (inverted) are shown. The quantification of the increase in CHOP-GFP intensity in time after DCF and CBZ exposure is presented as fold changes (FC) of time-point 0. (B) CHOP induction after carbamazepine and diclofenac exposure was followed in time by Western blotting. "C," control exposed to vehicle for 12 h. Tubulin was used as loading control. (C) Apoptosis induced by CBZ/TNFα after knockdown of CHOP (SMARTpool) was followed in time by automated imaging of AnxV-Alexa633 staining. Data are presented as means of three independent experiments ± SEM.

ducers of hepatotoxicity (Choi, 2003; Stricker *et al.*, 1986; West, 1997), have not, to our knowledge, been linked to immune system activation. In addition, supporting our results, absence of TNFα synergism with NFZ and MTX was previously reported in a larger screen of compounds in primary human hepatocytes with and without the addition of pro-inflammatory cytokines including TNFα (Cosgrove *et al.*, 2009). Moreover, the current manuscript illustrates the fact that whether or not a compound would synergize with TNFα may lie in the types of stress pathways induced by the drugs alone, where DCF and CBZ affect ER-stress/translation initiation signaling as well as oxidative stress, whereas NFZ and KTZ are stronger inducers of oxidative stress alone.

Our data indicate that ER stress signaling through the PERK/CHOP pathway is a critical determinant for the hepatotoxicant/TNFα synergy response toward hepatocyte apoptosis. ER stress and the UPR have been implicated in several different liver diseases including DILI (Dara *et al.*, 2011). Here we present a more selective activation of the PERK-arm of the ER stress/UPR following DCF and CBZ exposure (Figs. 5 and 6), which was directly related to expression of CHOP (Fig. 7), a downstream target of ATF4. Importantly, the up-regulation of ATF4 and CHOP was not only found in our HepG2 cell system after exposure of DCF and CBZ but also in primary human hepatocytes after exposure to a panel of hepatotoxic drugs (Fig. 3) as well as in other primary cell systems after DCF and CBZ exposure (Fig. 6B and Supplementary fig. S4). Moreover, in primary human hepatocytes, we found that many DILI compounds affected the expression of ATF4 and CHOP, whereas XBP1 and BiP/HSPA5 were less affected. Interestingly, this up-regulation of CHOP and ATF4 was more prominent with drugs related to severe DILI (Fig. 3). Given our observed critical role of CHOP in the onset of apoptosis, these combined observations suggest that CHOP is a critical player in liver toxicity, in particular in the sensitization for TNF receptor-mediated apoptosis.

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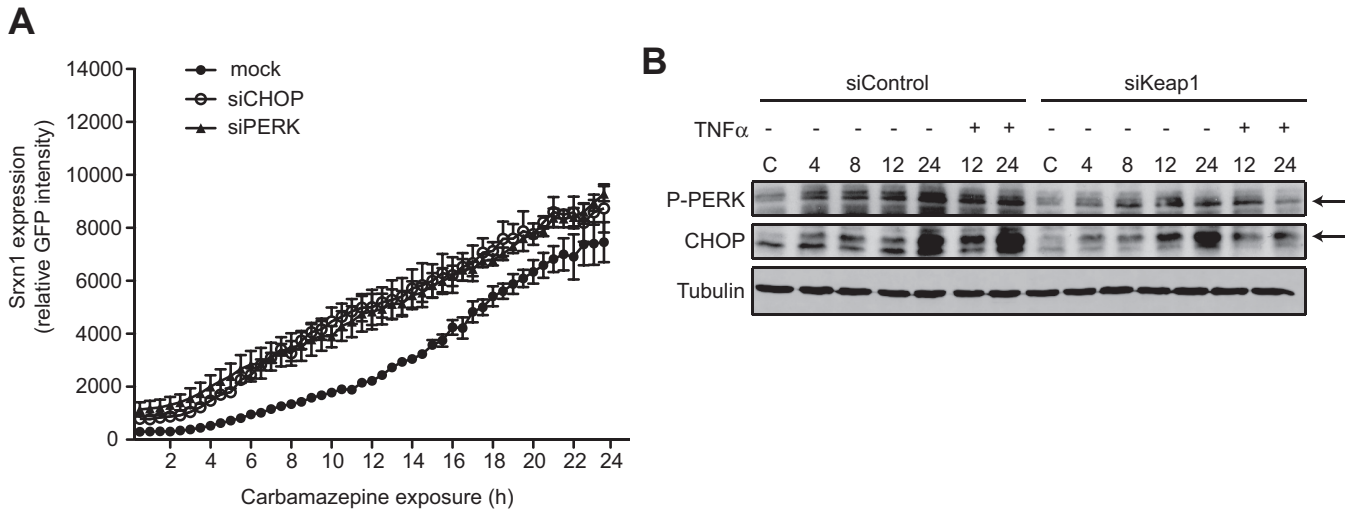

FIG. 8. Carbamazepine-induced Nrf2 activation is independent of ER stress. (A) The effect of PERK and CHOP knockdown on Srxn1-GFP induction after carbamazepine (CBZ; 500μM) exposure was investigated using automated confocal microscopy. GFP intensities were normalized to the area occupied by nuclei as determined by Hoechst staining. (B) ER-stress activation, as measured by protein expression of phosphorylated PERK and CHOP using Western blot, was investigated after Keap1 knockdown and a time series of CBZ exposure ± TNFα addition. Tubulin serves as loading control and "C" is control exposed to vehicle for 12 h. The data are presented as means of three independent experiments ± SEM or representative of three independent experiments.

The current study highlights the importance of perturbations in the translation initiation program in the hepatotoxicant-induced stress response and onset of cytotoxicity. Firstly, several translation initiation factors, which included EIF4A1, EIF4A2, and EIF4G3 were specifically strongly affected by CBZ and DCF but not the other hepatoxicants (Fig. 2B and Supplementary fig. S1A). Secondly, an inhibitor of eIF2α dephosphorylation, salubrinal, inhibited the drug/TNFα-induced apoptosis (Fig. 9A and Supplementary fig. S3D). Thirdly, knockdown of EIF4A1 almost completely abrogated the TNFα synergy with both CBZ and DCF (Fig. 9B and Supplementary fig. S3E). EIF4A1 and EIF4G3 together with cap-binding protein EIF4E are part of the EIF4F complex that unwinds secondary structures of the 5′ untranslated region (UTR) of mRNA to allow ribosomal binding, scanning, and thereby translation (Parsyan *et al.*, 2011). The 5′ UTR of mRNA can be more or less structured, determining its translation efficiency (Jackson *et al.*, 2010). EIF4A and EIF4G have also been implicated with cap-independent translation (Jackson *et al.*, 2010). Interestingly, the translation of several anti- and pro-apoptotic genes such as XIAP, and APAF1 can occur via cap-independent mechanisms (Coldwell *et al.*, 2000; Holcik *et al.*, 1999) and given the drastic effect on drug/TNFα-induced apoptosis we show (Fig. 9B and Supplementary fig. S3E), EIF4A1 is most likely involved in the expression of other apoptosis-regulating proteins. Here we present that EIF4A1 is a crucial regulator of pro-apoptotic CHOP expression because depletion of EIF4A1 reduced the expression of this protein (Fig. 9C), and CHOP protein expression is likely regulated by EIF4A1 cap-independent translation in our model. Of note is that also in the primary human hepatocytes various DILI compounds affected the expression of EIF4A1, EIF4A2, and/or EIF4G3 (Fig. 3). Although our combined results emphasize a role of translational control in xenobiotic toxicity, further research to uncover the entire (cap-independent) translation-based proteome will provide overall insight into the diversity of molecular networks that underlie the drug/TNFα synergy.

The hepatotoxicant/TNFα synergy was for an important part sensitized by the pro-oxidant properties of both CBZ and DCF (Fig. 4C and Supplementary fig. S2C). Indeed, our gene expression profiling showed strong up-regulation of Nrf2 target genes by both DCF and CBZ, which correlated with strong Nrf2-dependent induction of Srxn1 (Fig. 4B). Such an up-regulation of Srxn1 was also observed for human and mouse primary hepatocytes as well as human liver slices (data not shown), which fits with observations for the *in vivo* DCF treated rat liver (Deng *et al.*, 2008) as well as DCF treated mouse liver (Cantoni *et al.*, 2003). Importantly, knockdown of the endogenous Nrf2-inhibitor Keap1 led to protection against DCF/TNFα and CBZ/TNFα-induced apoptosis (Fig. 4C and Supplementary fig. 2C). In addition, hepatocyte cell death induced by DCF and CBZ alone is oxidative stress dependent (Gómez-Lechón *et al.*, 2003; Santos *et al.*, 2008). Despite the fact that Keap1 knockdown was strongly protective against drug/TNFα synergy, it did not affect PERK activation and CHOP expression (Fig. 8B). This suggests that the drug-induced ER stress/UPR is uncoupled from oxidative stress, and that both stress programs each independently modulate the susceptibility toward TNFα-mediated synergistic drug induced cell killing.

It is of crucial importance to elucidate the connection between ER stress/UPR and the onset of apoptosis by the drug/TNFα combinations. TNFα itself or in combination with CBZ or DCF

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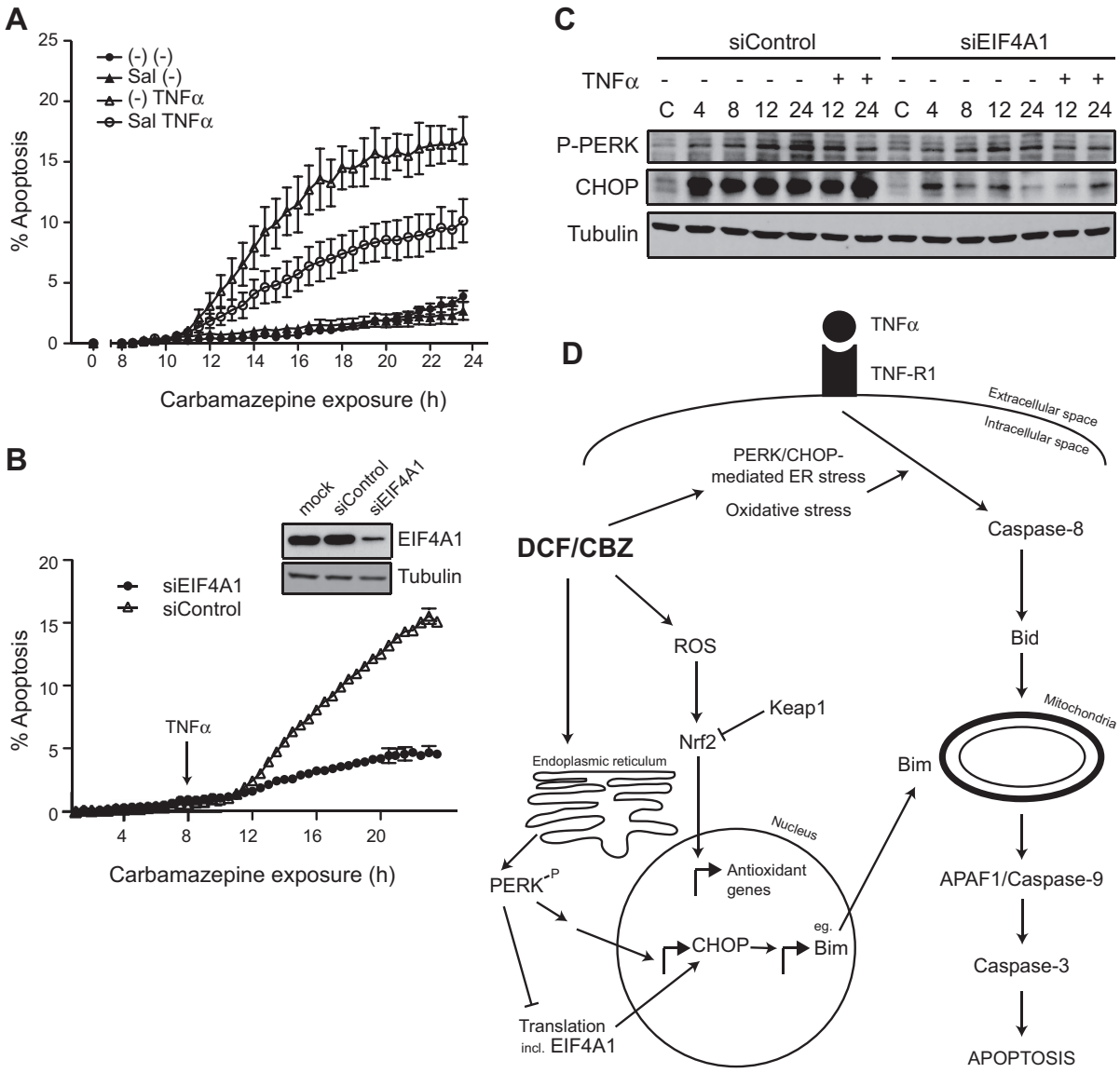

FIG. 9. Translation initiation regulated by EIF4A1 is crucial for carbamazepine/TNFα-apoptosis induction and CHOP expression. (A) HepG2 cells were pre-treated with an eIF2α phosphatase inhibitor, salubrinal (Sal; 50μM), for 16 h before treatment with 500μM carbamazepine (CBZ). TNFα (10 ng/ml) was added 8 h after drug exposure. (B) Apoptosis induced by CBZ (500μM) and TNFα (10 ng/ml) after EIF4A1 knockdown (SMARTpool) was followed in time using live cell imaging of apoptosis. (C) The effect of EIF4A1 knockdown on the induction of ER-stress proteins P-PERK and CHOP by Western blot. The induction of caspase-8 cleavage was used as a marker of death receptor induced apoptosis activation. Tubulin served as loading control. All data are presented as means of three independent experiments ± SEM or representative for three independent experiments when applicable. (D) Model of the molecular mechanisms of diclofenac (DCF) and CBZ-induced sensitization toward TNFα-induced apoptosis.

did not enhance CHOP protein levels (Fig. 7B), therefore enhancement of CHOP expression is not the sole mechanism behind the enhanced apoptosis observed upon TNFα addition to CBZ and DCF pre-exposed cells. Rather, it seems as if drug-induced CHOP expression leads to sensitization of the HepG2 cells to TNFα-induced apoptosis, and because CHOP is a transcription factor, it is likely that downstream target genes is what affects cell susceptibility. Up-regulation of CHOP may lead to apoptosis via up-regulation of pro-apoptotic Bcl-2 family members, including Bim (Puthalakath *et al.*, 2007). In our system, Bim (BCL2L11) was up-regulated after DCF and CBZ exposure and Bim is induced by different DILI compounds in primary hepatocytes in close association with CHOP expression (Figs. 2 and 3) further emphasizing their close connection. Moreover, siRNA-mediated knockdown of Bim rescued both the CBZ/TNFα- and DCF/TNFα-induced cytotoxicity (Supplementary table S1; Fredriksson *et al.* (2011)). In addition to the role of Bim, our current data demonstrate that the CBZ/TNFα-

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induced apoptosis was inhibited by knockdown of caspase-8, Bid, APAF1, caspase-9, and caspase-3 (Supplementary table S1). Together these data suggest that drug-induced Bim expression sensitizes the mitochondria to caspase-8-mediated Bid cleavage and activity, causing the synergistic apoptosis induction with TNFα. We are currently investigating the role of other proteins in this synergistic apoptotic response using an unbiased siRNA screening approach.

In summary, we show that DCF and CBZ, drugs linked to idiosyncratic DILI with activation of the inflammatory system, sensitize liver cells to TNFα-induced apoptosis. We propose an overall working model (Fig. 9D) where CBZ and DCF induce oxidative and ER stress/UPR, which independently sensitize toward apoptosis. The PERK/ATF4/CHOP-dependent ER stress/UPR program enhances the activation of the apoptotic signaling downstream of the TNF receptor in close control by the translation initiation program including EIF4A1. Subsequent expression and/or activation of caspase-8, Bid, and Bim drive the activation of the intrinsic apoptotic program controlled by APAF1 and caspase-9 to activate the onset of caspase-3. Our work sheds new light on the mechanism behind the, so far, unpredictable idiosyncratic DILI. Possibly genetic variants in the functionally critical determinants of the cytotoxic response are candidate susceptibility genes that predispose for individual humans to idiosyncratic DILI.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org.

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