J. Biochem. 2009;146(1)123–132
doi:10.1093/jb/mvp052

Gene Expression Profiling Identifies a Role for CHOP During Inhibition of the Mitochondrial Respiratory Chain

Fumihiro Ishikawa[1], Takashi Akimoto[1], Haruka Yamamoto[1], Yuri Araki[1], Toshihiko Yoshie[1], Kazunori Mori[1], Hidetoshi Hayashi[2], Kiyoshi Nose[1] and Motoko Shibanuma[1,*]

[1]Department of Microbiology, Showa University School of Pharmacy, Tokyo 142-8555; and [2]Department of Molecular Health Sciences, Graduate School of Pharmaceutical Sciences, Nagoya City University, Mizuho-cho, Nagoya, Aichi, Japan

Received December 29, 2008; accepted March 7, 2009; published online March 20, 2009

Mitochondrial dysfunction, in particular, interference in the respiratory chain, is often responsible for the toxicogenic effects of xenobiotics. In this study, changes in gene expression resulting from pharmacological inhibition of the respiratory chain were studied by DNA microarray analysis using cells treated with rotenone or antimycin A, which inhibit complexes I and III of the electron transport system, respectively. Forty-eight genes were either up- or down-regulated more than 3-fold. These included stress- and/or metabolic-related effector genes and several transcriptional regulators represented by CHOP-10. Further study using siRNA showed that among the four genes studied, up-regulation of three was dependent on CHOP-10. C/EBPβ, a dimerizing partner of CHOP-10, was also involved in two of the three genes including Trib3, implying that CHOP-10, heterodimerizing with C/EBPβ or another partner played a key role in the expression of a set of genes under stress. Although *CHOP-10* and *Trib3* were both ER-stress response genes, signal inducing Trib3 during mitochondrial stress was distinct from that during ER stress. Cytotoxicity caused by inhibition of the respiratory chain was attenuated by treatment with siRNA for CHOP-10. This study demonstrated the importance of CHOP-10 in coordinating individual gene expression in response to the mitochondrial stress.

Key words: C/EBPβ, CHOP-10, DNA microarray, mitochondrial stress, respiratory chain.

Abbreviations: CHOP, CCAAT/enhancer-binding protein (C/EBP) homology protein-10; C/EBP, CCAAT/enhancer-binding protein; ER, endoplasmic reticulum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT–PCR, reverse transcription-polymerase chain reaction; Trib3, *tribbles*-related protein 3.

Mitochondria are unique and valuable targets for xenobiotics. In particular, the presence of different organellar sub-compartments enable unique distribution of chemicals and mitochondrial DNA provides prominent target sites, thereby emphasizing that mitochondria acts as distinctive organelles in pharmacotoxicology (*1*). Till date, a diverse array of chemicals have been reported that directly or indirectly interact with specific components of mitochondria, including mitochondrial DNA (mtDNA), and thus, exert therapeutic, toxic and/or pharmacological effects on cells. Mitochondrial function is extremely complex and encompasses catabolic and anabolic pathways, redox balance, cell death and differentiation, and mitosis in addition to specialized cell functions that include calcium homeostasis, thermogenesis, signalling via reactive oxygen species and nitric oxide species, ion channel regulation and metabolite transport. Therefore, mitochondrial dysfunction is inevitably associated with a variety of pathophysiological syndromes such as lactic acidosis, myopathy, neuropathy, retinopathy and enteropathy (*2*).

Among potential chemical targets, the mitochondrial respiratory chain composed of four multi-enzyme complexes I–IV has been the focus of many studies because of its critical role in supplying ATP and its close association with other essential mitochondrial functions. Numerous specific molecules have been reported to inhibit these complexes. Rotenone is a representative of complex I inhibitors, and antimycin A and oligomycin are specific for complexes III and IV, respectively (*3*). Along with specific inhibitors of these enzymatic complexes, chemicals that induce promiscuous mutations, deletions, or depletion of mtDNA also interfere with the activity of the respiratory chain. This is because mtDNA encodes 13 polypeptides, 22 transfer RNAs and 2 ribosomal RNAs, all of which are essential for the synthesis of enzymatic components of the respiratory chain. For example, nucleotide reverse transcriptase inhibitors such as zalcitabine and didanosine have recently been demonstrated to inhibit mitochondrial-specific DNA polymerase γ, which leads to progressive depletion of mtDNA (*4*, *5*).

For prevention or alleviation of toxicity, as well as for *bona fide* evidence-based therapeutic applications of chemicals, understanding the molecular mechanisms

*To whom correspondence should be addressed: Tel: +81-3-3784-8209, Fax: +81-3-3784-6850, E-mail: smotoko@pharm.showa-u.ac.jp

underlying the pharmacological effects is crucial. Therefore, the aim of this study is to understand cellular responses upon disruption of the mitochondrial respiratory chain at the molecular level. To accomplish this, gene expression profiles from the mouse whole genome were used to identify genes that were up- or down-regulated by inhibitors of complexes I and III. Along with metabolic- and stress-related genes, transcriptional regulators such as CHOP-10 (CCAAT/enhancer-binding protein (C/EBP) homology protein-10) were identified in the profile. In particular, CHOP-10 regulated the expression of a subset of genes included in the same profile. Thus, we identified a hierarchical transcriptional cascade underlying cellular responses during mitochondrial stress in which an upstream transcriptional regulator controlled downstream target genes.

MATERIALS AND METHODS

Cell Culture and Chemicals—Mouse mammary epithelial NMuMG cells were obtained and maintained as described previously (*6*). Mouse C2C12 skeletal myoblasts and 3T3-L1 fibroblasts were grown in Dulbecco's modified MEM supplemented with 15% and 10% fetal calf serum, respectively. Rotenone and antimycin A were obtained from Sigma (St Louis, MO).

DNA Microarray—DNA microarray analysis was performed following the Two-Color Microarray Gene Expression Analysis protocol (Agilent Technologies, Santa Clara, CA). Total RNA, isolated from the cells described above, was reverse-transcribed, and 3′- or 5′-cyanine-labelled cRNA was synthesized using the Low RNA Input Linear Amplification Kit Plus, Two Color (Agilent Technologies). Equal amounts of cRNA samples per pair were hybridized to whole mouse genome (4×44 K) Oligo Microarray chips (Agilent Technologies). Microarray scanning was performed using a GenePix 4000B scanner (Agilent Technologies), and image and statistical analyses of primary spot intensity were performed using Agilent's Feature Extraction Software.

Antibodies and Western Blotting—Monoclonal anti-CHOP-10 (GADD153) and polyclonal anti-C/EBPβ were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody was obtained from Chemicon (Temecula, CA). The procedure for Western blotting was essentially the same as described before (*7*).

siRNA and Transfection—Two siRNAs (small interfering RNA) each for mouse CHOP-10 and C/EBPβ were designed and purchased from Nippon EGT (Toyama, Japan). The negative control was purchased from GE Healthcare (Buckinghamshire, UK). The sequences for CHOP-10 were as follows: #1 (453S/AS) 5′-GCAAGG AAGAACUAGGAAATT-3′ and 5′-UUUCCUAGUUCUUC CUUGCTT-3′, and #2 (576S/AS) 5′-GCGGCUCAAGCAG GAAAUCTT-3′ and 5′-GAUUUCCUGCUUGAGCCGCTT-3′; and for C/EBPβ #1 (766S/AS) 5′-AGCUGAGCGACGA GUACAATT-3′ and 5′-UUGUACUCGUCGCUCAGCUTT-3′, and #2 (937S/AS) 5′-CCCUGCGGAACUUGUUCA ATT-3′ and 5′-UUGAACAAGUUCCGCAGGGTT-3′. Cells were transfected with siRNA duplexes (100′nM) using Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer's instructions. At 48 h post-transfection, the cells were processed for analysis.

Real-Time Reverse Transcription Polymerase Chain Reaction (RT–PCR)—Total RNA from cultured cells was extracted using the Quickgene RNA Cultured Cell Kit (Fuji Photo Film Co., Ltd, Tokyo, Japan), and cDNA was synthesized from 1 μg RNA with Primescript reverse transcriptase (Takara Shuzo, Kyoto, Japan). Aliquots of cDNA samples were mixed with SYBR Green PCR Master Mix (Nippon Gene Co., Ltd, Toyama, Japan) and 0.5 μM of each primer, and loaded onto the MyiQ Real-time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA). After incubation for 10 min at 95°C, the mixtures were subjected to 40 amplification cycles (20 s at 94°C for denaturation, 20 s at 55°C for annealing and 30 s at 72°C for extension). The quantities of the respective mRNAs were normalized using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA as a control.

The following primers were used: ATF3 (forward, 5′-GGCGGCGAGAAAGAAATAAA-3′; reverse, 5′-CAGGCAC TGTCTTCTCCTTTTT-3′); CHOP-10 (forward, 5′-TACTC TTGACCCTGCGTCCCTA-3′; reverse, 5′-TGACTGGAAT CTGGAGAGCGA-3′); C/EBPβ (forward, 5′-ATCCGGATC AAACGTGGCT-3′; reverse, 5′-AACCCCGCAGGAACATC TTTA-3′); E2F7 (forward, 5′-CGTCTCTGCATCCATCT TACCA-3′; reverse, 5′-CAGCCTCTCTTTTGCACACACT-3′); Zhx1 (forward, 5′-ACAGACACAAAACCAGCCACAG-3′; reverse, 5′-GGCCCGAATACCAAATGAATC-3′); p107 (forward, 5′-TGATAAGGCAAGGCGAGCA-3′; reverse, 5′-AGAGACGTTTGGCAGGTGAGTC-3′); TRB3 (forward, 5′-CAGTCCCTTTTATCAGTGCCCC-3′; reverse, 5′-GATT TGTGGTCCTGAGGCACAT-3′); Gadd45a (forward, 5′-CA GATCCATTTCACCCTCATCC-3′; reverse, 5′-TCCAGTAG CAGCAGCTCAGCTA-3′); Gsta1 (forward, 5′-AGAAGTT CCTACAGCCTGGCAG-3′; reverse, 5′-GAAAGCCTTCCT TGCTTCTTGA-3′); Ptgs2 (forward, 5′-TGACCCCCAAG GCTCAAATA-3′; reverse, 5′-ACCCAGGTCCTCGCTTAT GAT-3′); and GAPDH (forward, 5′-ATGTGTCCGTCGTG GATCTGA-3′; reverse, 5′-ATGCCTGCTTCACCACCTT CT-3′).

Luciferase Assay—Cells were transfected with reporter plasmids, a series of pTRB3-Luc constructs (*8*), or the control plasmid, pGL3 (Promega, Madison, WI), together with an internal control plasmid, pRL/CMV, using Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer's instructions. Luciferase activity was quantified 24 h after transfection using the Dual Luciferase Assay Kit (Promega). Each assay was performed in duplicate and repeated at least three times, and the value was corrected using *Renilla* luciferase activity expressed from the internal control plasmid.

Cell Viability and Cytotoxicity Assay—Cell viability was assessed by exclusion of trypan blue or by tetrazolium (MTT) assay in which mitochondrial reductase activity was monitored to reflect the viability of cells. Cells were cultured in 96-well plates with or without chemicals. Ten microliters of MTT solution (5 mg/ml in PBS) was added to 100 μl of culture medium and incubated for 4 h at 37°C to reduce MTT to formazan by mitochondrial reductase. The product was extracted

with 100 μl isopropanol containing 40 mM HCl, and absorbance was measured at 570 nm.

RESULTS

DNA Microarray Analysis Identifies Genes that are Up- and Down-Regulated in Response to Inhibition of the Mitochondrial Respiratory Chain—Mouse whole-genome DNA microarray analysis was performed to obtain genome-wide profiles of differentially expressed genes in experimental cell models in which the mitochondrial respiratory chain was inhibited pharmacologically. Mouse mammary epithelial NMuMG cells were treated with rotenone, a well-established inhibitor of complex I, or antimycin A, an inhibitor of complex III, at maximal doses where the cell survival rate was greater than 75% following 24 h treatment (Fig. 6A). Total RNA was extracted from sample pairs, either treated or untreated, copied into cDNA by reverse-transcription, followed by the synthesis of 3′- or 5′-cyanine-labelled cRNA. Statistical analysis of the fluorescence intensities of the spots produced by hybridization of the differentially labelled cRNA mixtures on the microarray identified 48 genes that showed more than a 3-fold up- or down-regulation by treatment with antimycin A and rotenone, suggesting that the change was in response to inhibition of the respiratory chain and not due to side-effects inherent to each chemical. As listed in Table 1, 33 genes were up-regulated and 15 down-regulated by chemical treatment. Of these, 10 genes were examined by real-time RT–PCR, and their expression pattern was the same as the expression patterns obtained with DNA microarray, thereby confirming the results of the microarray (Fig. 1).

The disruption of the mitochondrial respiratory chain varies from cell-type to cell-type, depending on the inherent requirement for high aerobic energy. In addition to the NMuMG epithelial cells, gene expression was examined by real-time RT–PCR in C2C12 myoblasts and 3T3-L1 preadipocytes treated with the same pharmacological inhibition conditions of the mitochondrial respiration. As expected, differences were observed between the three different cell lines. The expression patterns of genes *E2F7*, *p107* and *Zhx1* provide an excellent example of this (Fig. 1). The expression of these transcriptional regulators was repressed in the epithelial cells (NMuMG) and myoblasts (C2C12), whereas it was unchanged, or actually increased, in the 3T3-L1 preadipocytes (Fig. 1). The response of Mmp13 was markedly higher in NMuMG cells (Fig. 1).

Upstream Transcriptional Regulators Control Downstream Effector Genes in a Hierarchical Regulatory Cascade in Response To Mitochondrial Stress—A set of transcriptional regulators were noted in the list of genes up- or down-regulated by the mitochondrial stress along with the genes that were stress- and/or metabolic-related effectors (Table 1). For example, *CHOP-10* (*DDIT3*) and *Atf3* were up-regulated, whereas *Zhx1*, *E2F7 and Rbl1* (*p107*) were down-regulated. Therefore, the possibility that these genes were involved in the regulation of effector genes was investigated. In this study, we focused on *CHOP-10*, a stress-inducible transcriptional regulator, which is also known as growth arrest- and DNA damage-inducible 153, DNA-damage-inducible transcript 3 (*DDIT3*) and *C/EBPζ* (*9*). *CHOP-10* has been shown to dimerize with members of the C/EBP family of transcription factors and act as either a dominant-negative regulator of C/EBP proteins for classic C/EBP target genes or as a transcriptional activator for more recently identified CHOP-C/EBP target genes (*10*). We examined its role in gene expression under mitochondrial stress elicited by rotenone in C2C12 cells. It is noteworthy that the up-regulation of *CHOP-10* was clearly observed at the protein level and mRNA level in this cell line under mitochondrial stress (Figs 2A and 3).

To investigate the role of *CHOP-10* (CHOP), CHOP siRNA was introduced into the cells, and effects on gene expression were examined. In parallel experiments, the involvement of C/EBPβ, a major heterodimer partner of CHOP, was also examined. As shown in Figs 2B and 3, two different siRNAs, each with unrelated sequences (labelled #1 and #2) for each gene product, reduced the expression of *CHOP* and *C/EBP*β genes, respectively, at a protein as well as the mRNA level.

Among the transcriptional regulators tested, the *ATF3* expression was appreciably induced by stress, and this induction was clearly attenuated by treatment of cells with CHOP siRNAs (Fig. 3). This result suggests that *CHOP*, which is up-regulated by stress (Figs 1, 2A and 3), regulates other stress-response transcriptional regulators such as *Atf3* in the cells. The down-regulation of other transcription factors, such as *E2F7*, *Zhx1* and *p107*, by stress is unaffected by siRNAs, while the basal expression levels of *E2F7* and *p107* were reduced by CHOP siRNA treatment, implicating that CHOP is involved in the regulation of the expression of the two genes in normal cells (Fig. 3, open columns; control). In contrast, the effect of the C/EBPβ siRNAs on expression of these transcriptional regulators was marginal with low statistical significance (Fig. 3).

The effects of siRNA treatment on the induction of the stress-related effector genes, *Trib3*, *Gsat1 and Gadd45a*, were also observed. As shown in Fig. 4, rotenone induction of *Trib3* in C2C12 cells, which was the most prominent response to mitochondrial stress, was inhibited by treatment with CHOP and C/EBPβ siRNAs. Likewise, induction of *Gsta1* was also clearly attenuated by CHOP and C/EBPβ siRNAs. These results suggest that prominent induction of *Trib3* and *Gsta1* in response to mitochondrial stress is dependent on CHOP and C/EBPβ. These observations suggest that CHOP and C/EBPβ are key regulators and affect the gene expression pattern under mitochondrial stress conditions. In contrast to *Trib3* and *Gsta1*, induction of *Gadd45* was unaffected by treatment with CHOP and C/EBPβ siRNAs, suggesting that the induction of *Gadd45a* is independent of CHOP and C/EBP.

Regulation of Trib3 Induction Under Mitochondrial Stress was Distinct from that Observed in ER stress—In addition to mitochondrial stress as demonstrated in Figs 1 and 2; *Trib3* and *CHOP* are induced following endoplasmic reticulum (ER) stress (*8*, *11*). Given that the induction of *Trib3* under ER and mitochondrial stress

Table 1. **Microarray analysis of changes in gene expression during mitochondrial stress.**

Gene ID	Official gene name (Symbol)	Antimycin A	Rotenone	Ave
Up-regulation				
12353	Carbonic anhydrase 6 (Car6)	25.81	24.81	25.31
17386	Matrix metalloproteinase 13 (Mmp13)	29.61	11.86	20.74
12862	Cytochrome *c* oxidase, subunit VI a, polypeptide 2 (Cox6a2)	10.13	13.93	12.03
13197	Growth arrest and DNA-damage-inducible 45 alpha (Gadd45a)	14.59	7.3	10.94
13198	DNA-damage inducible transcript 3 (Ddit3)	11.61	9.17	10.39
12496	Ectonucleoside triphosphate diphosphohydrolase 2 (Entpd2)	14.61, 15.56	5.49, 4.33	10
56213	HtrA serine peptidase 1 (Igf binding) (Htra1)	6.94	10.72	8.83
19225	Prostaglandin-endoperoxide synthase 2 (Ptgs2)	12.64	3.97	8.3
70726	Angiopoietin-like 6 (Angptl6)	12.11	3.97	8.04
22421	Wingless-related MMTV integration site 7A (Wnt7a)	8.26	7.3	7.78
17872	Myeloid differentiation primary response gene 116 (Myd116)	8.04	7.46	7.75
216188	Aldehyde dehydrogenase 1 family, member L2 (Aldh1l2)	10.06	4.95	7.51
50527	ERO1-like (S. cerevisiae) (Ero1l)	9.82	5	7.41
20583	Snail homolog 2 (Drosophila) (Snai2)	10.16	4.63	7.39
56312	Nuclear protein 1 (Nupr1)	9.97	4.65	7.31
17988	N-myc downstream regulated gene 1 (Ndrg1)	8.6	5.41	7
11997	Aldo-keto reductase family 1, member B7 (Akr1b7)	5.72	7.3	6.51
93732	Acyl-Coenzyme A oxidase 2, branched chain (Acox2)	6.06	6.94	6.5
103172	Nur77 downstream gene 2 (Ndg2)	6.28	6.62	6.45
14528	GTP cyclohydrolase 1 (Gch1)	8.75	4.12	6.43
12514	CD68 antigen (Cd68)	6.89	5.71	6.3
228775	Tribbles homolog 3 (Drosophila) (Trib3)	6.28, 5.29	6.99, 5.13	5.92
12176	BCL2/adenovirus E1B 19kDa-interacting protein 1, NIP3 (Bnip3)	8.59	3.24	5.91
24111	Urotensin 2 (Uts2)	6.69	3.73	5.21
228775	Tribbles homolog 3 (Drosophila) (Trib3)		5.13	5.13
107869	Cystathionase (cystathionine gamma-lyase) (Cth)	4.45	5.68	5.07
11910	Activating transcription factor 3 (Atf3)	5.52	4.03	4.78
16421	Integrin beta 7 (Itgb7)	5.38	3.62	4.5
23959	5′ nucleotidase, ecto (Nt5e)	5.47	3.53	4.5
14857	Glutathione S-transferase, alpha 1 (Ya) (Gsta1)	3.48	4.52	4
18412	Sequestosome 1 (Sqstm1)	4.77	3.11	3.94
14858	Glutathione S-transferase, alpha 2 (Yc2) (Gsta2)	3.46	3.89	3.67
223881	Rho family GTPase 1 (Rnd1)	3.5	3.34	3.42
Down-regulation				
107581	Procollagen, type XVI, alpha 1 (Col16a1)	0.14	0.24	0.19
12490	CD34 antigen (Cd34)	0.14	0.28	0.21
81877	Tenascin XB (Tnxb)	0.22	0.21	0.21
22770	Zinc fingers and homeoboxes protein 1 (Zhx1)	0.16	0.28	0.22
20135	Ribonucleotide reductase M2 (Rrm2)	0.14	0.31	0.22
18186	Neuropilin 1 (Nrp1)	0.24	0.22	0.23
52679	E2F transcription factor 7 (E2f7)	0.22	0.24	0.23
228911	Teashirt zinc finger family member 2 (Tshz2)	0.26	0.23	0.24
19041	Periplakin (Ppl)	0.25	0.25	0.25
15894	Mouse (clone lambda-c5e) intercellular adhesion molecule 1 (ICAM-1)	0.26	0.3	0.28
19650	Retinoblastoma-like 1 (p107) (Rbl1)	0.31	0.3	0.3
78651	LSM6 homolog, U6 small nuclear RNA associated (S. cerevisiae) (Lsm6)	0.3	0.32	0.31
14897	Thyroid hormone receptor interactor 12 (Trip12)	0.31	0.3	0.31
14164	Fibroblast growth factor 1 (Fgf1)	0.3	0.33	0.31
384247	Hypothetical protein E130307C13 (E130307C13)	0.32	0.32	0.32

NMuMG cells were treated with rotenone (5 nM) or antimycin A (9 nM), or vehicle for 24 h and total RNA was extracted. Two independent pairs (treated and non-treated) of the cRNA pool were generated using reverse-transcribed RNA as a template and hybridized with a 4 × 44 K whole mouse genome microarray. The analysis was performed with GeneSpring as described in MATERIALS AND METHODS section. The average of the duplicate hybridization for each gene is shown. Ave denotes the average of rotenone- and antimycin A-treated samples.

conditions was dependent on *CHOP* (Fig. 4) (*8*), it is possible that *Trib3* induction is under the same regulatory control in both stress conditions, providing an example of cross-talk between the two stress signalling cascades. This idea was tested using a luciferase reporter that included the Trib3 promoter region harboring the CHOP-responsive element, and thus, responsive to ER stress (*8*). Four experimental reporter constructs were tested, pTRB3-Luc1, 2, 6 and 7, together with the control pGL3 plasmid. The reporter constructs, except

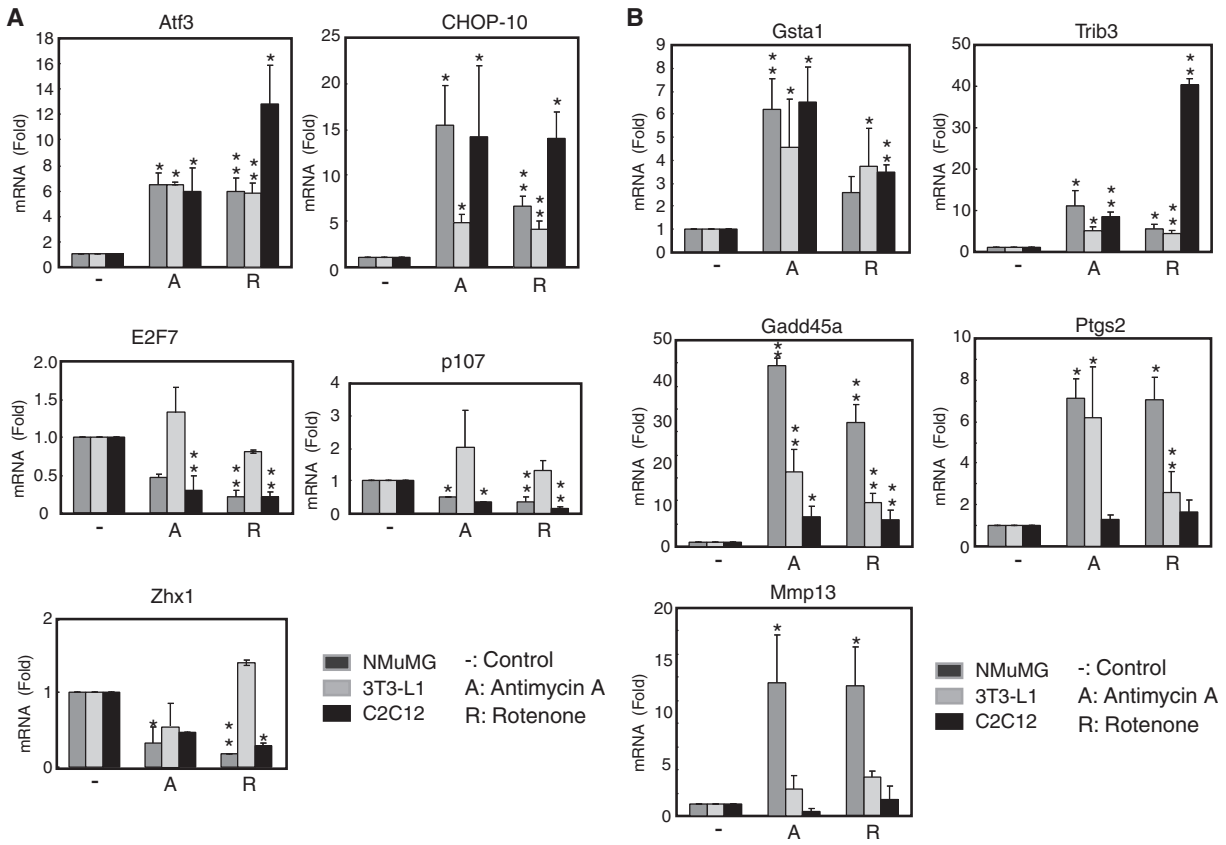

Fig. 1. **Changes in gene expression levels of specific transcriptional regulators and those of metabolic- and stress-related genes following treatment of cells with mitochondrial respiratory chain complex inhibitors.** NMuMG, 3T3-L1, or C2C12 cells were treated with antimycin A (9 nM), rotenone (5 nM), or vehicle for 24 h. Total RNA was extracted from the cells and the cDNA produced by reverse transcription was subjected to real-time PCR using primers specific to the coding region of each target gene (MATERIALS AND METHODS section). The mRNA quantities were normalized with that of GAPDH, and the values relative to the untreated samples are shown. The values are mean ± SD obtained from three independent experiments. (A) Transcriptional regulators. (B) Metabolic- and stress-related genes. The significance of the difference was assessed by *t*-test ($^{**}P < 0.005$ and $^{*}P < 0.05$).

pTRB3-Luc7 with a deleted ER-stress response element, showed a clear response to tunicamycin, an ER stress inducer. However, none of the reporter constructs responded to rotenone and antimycin A, suggesting that induction of *Trib3* under mitochondrial and ER stress conditions are under the control of two different promoter elements (Fig. 5). Therefore, it is likely that the signal that induces *Trib3* during mitochondrial stress is distinct from that which responds to ER stress, although *Trib3* is induced in a CHOP-dependent manner under both stress conditions. Consistent with this observation, among the seven genes that were known to be induced by CHOP in ER stress (Bcl2l11 and Ero1l) or responsive to ER stress [Atp2a2, Calr, Ddit3 (CHOP), Hspa5 and Hsp90b1], only two, Ddit3 and Ero1l, responded to mitochondrial stress (Table 2), suggesting that ER stress was not induced by inhibition of the mitochondrial respiratory chain.

Finally, we evaluated the contribution of CHOP in the induction of cell death, the final cellular response during mitochondrial stress. As shown in Fig. 6A, exposure of cells to rotenone and antimycin A decreased the cell viability in a dose-dependent manner as observed by the MTT assay. When cells were treated with the two

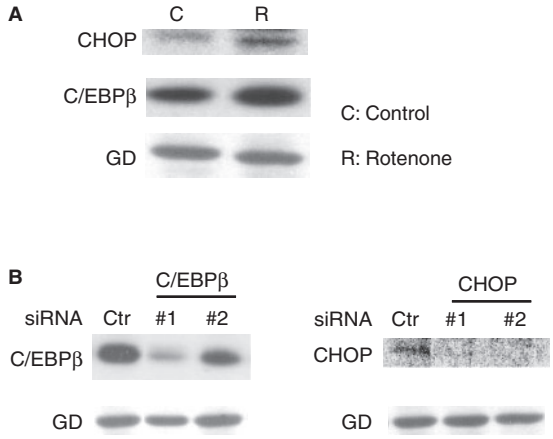

Fig. 2. **CHOP and C/EBPβ expression and their knock-down by siRNA at the protein level.** (A) C2C12 cells were treated with rotenone (5 nM) or vehicle as in Fig. 1, and the cell lysate was analyzed by western blotting using the specific antibodies for CHOP and C/EBPβ. (B) The cells were transfected with siRNA duplexes (100 nM) with two unrelated sequences (#1 and #2) for CHOP or C/EBPβ. At 48 h post-transfection, cells were lysed and subject to Western blotting. GD (GAPDH) is used as a loading control.

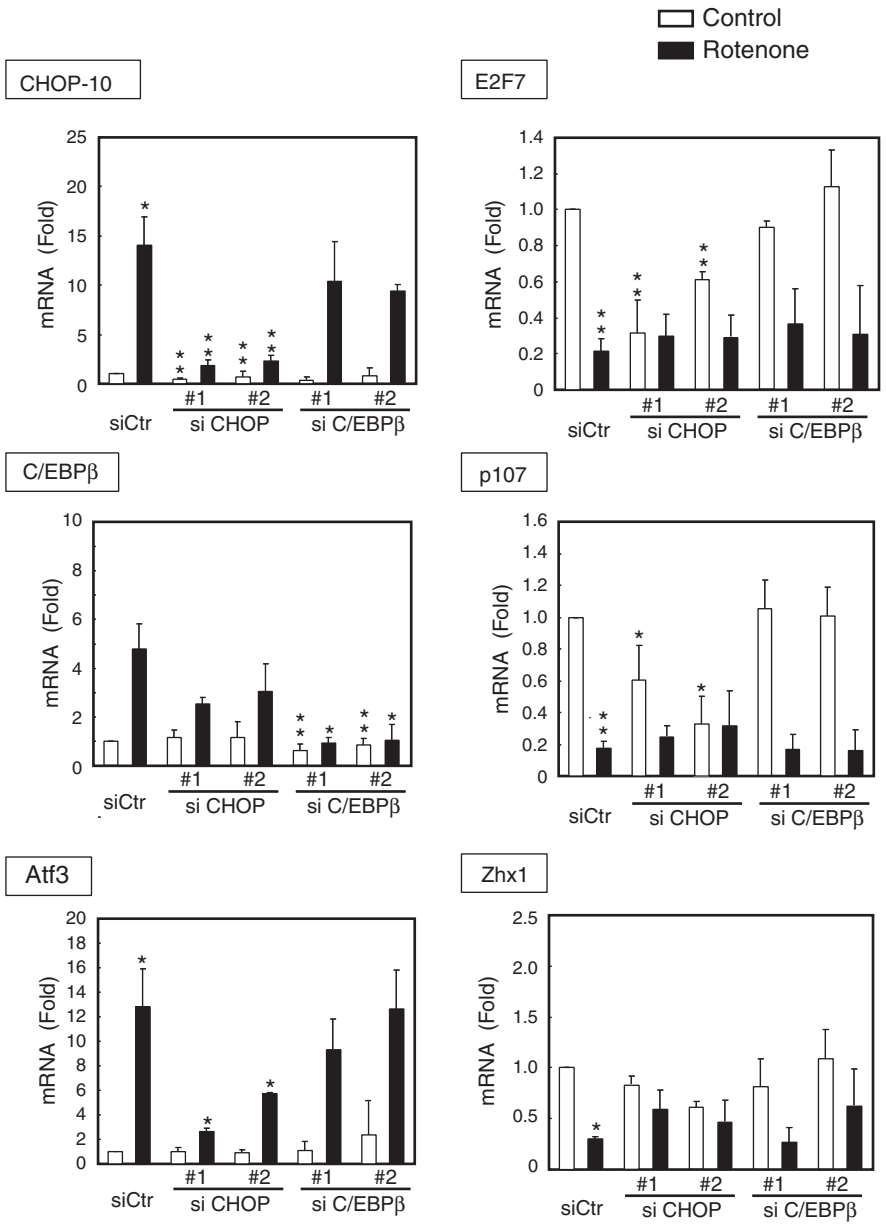

Fig. 3. **Effect of CHOP and C/EBPβ knockdown by siRNA on the expression of transcriptional regulatory genes under mitochondrial stress induced by inhibition of the respiratory chain.** C2C12 cells were transfected with siRNA duplexes (100 nM) with two unrelated sequences (#1 and #2) for CHOP or C/EBPβ. At 48 h post-transfection, cells were exposed to rotenone (5 nM) or vehicle for 24 h and processed by real-time RT–PCR as above. The quantities of the respective mRNAs were normalized with that of GAPDH, and values relative to the untreated samples are shown. The values are mean ± SD obtained from three independent experiments. The significance of the difference was assessed by *t*-test (**$P < 0.005$ and *$P < 0.05$).

inhibitors at lethal doses in the presence and absence of siRNA for CHOP, cell death assessed by the trypan blue exclusion assay was significantly alleviated by the knockdown of CHOP (Fig. 6B). Cell death was also detected by a method based on the protease activity released into the medium, and essentially the same effect of CHOP siRNA was observed (data not shown). SiRNA for C/EBPβ, on the other hand, had only a marginal effect on cytotoxicity (Fig. 6B). Given that cell death was accompanied by release of the protease into the medium and that strand breaks within the DNA and the activation of caspase-3 were not detected under mitochondrial stress (data not shown), it is likely that the induced cell death was necrotic.

DISCUSSION

For better understanding of the cellular response and underlying signalling pathways activated by mitochondrial dysfunction, gene expression during inhibition of the respiratory chain was profiled using a mouse genome oligo microarray. Recently, a similar analysis conducted

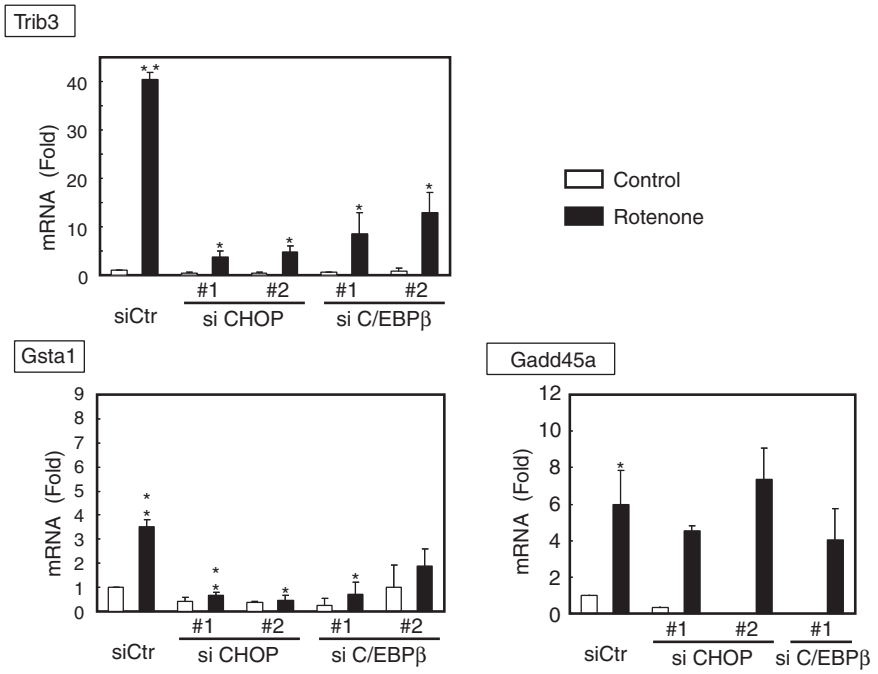

Fig. 4. **Effect of CHOP-10 and C/EBPβ knockdown by siRNA on the expression of metabolic- and stress-related genes during mitochondrial stress induced by inhibition of the respiratory chain.** C2C12 cells were transfected with siRNA, treated with antimycin A (9 nM) and rotenone (5 nM), and analyzed by real-time RT–PCR as above. The quantities of the respective mRNAs were normalized with that of GAPDH, and values relative to the untreated samples are shown. The values are mean ± SD obtained from three independent experiments. The significance of the difference was assessed by *t*-test (**$P < 0.005$ and *$P < 0.05$).

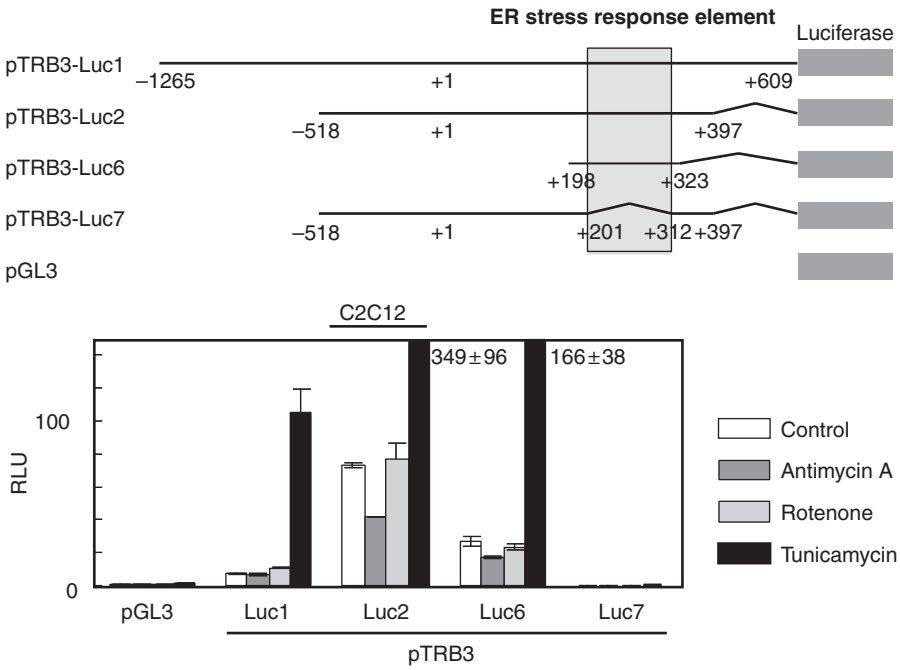

Fig. 5. **Responses of TRB3 promoter-luciferase reporters to ER and mitochondrial stresses.** C2C12 cells were transiently transfected with one of the reporter plasmids, pTRB3-Luc1, 2, 6 and 7, or the control plasmid pGL3, together with the internal control plasmid pRL/CMV. After 24 h, the cells were treated with antimycin A (9 nM), rotenone (10 nM) and tunicamycin (2 μg/ml) or vehicle. Luciferase activity was measured after another 24 h incubation as described in MATERIALS AND METHODS section. Each assay was performed in duplicate and repeated at least three times and the values relative to that of pGL3 from the untreated sample are shown after correction with the *Renilla* luciferase activity.

Table 2. **ER stress-related gene expression during mitochondrial stress.**

Official gene name (symbol)	Antimycin A	Rotenone
ATPase, Ca^{++} transporting, cardiac muscle, slow twitch2 (Atp2a2)	0.6945	0.5905
BCL2-like 1l (Bcl2l1l)	0.0245	1.39
Calreticulin (Calr)	1.088	0.723
DNA-damage inducible transcript 3 (Ddit3)	9.183	10.7
ERO1-like (Ero1l)	4.125	9.74
Glucose-regulated protein, 78 kDa (Hspa5)	1.51	1.49
Heat shock protein 90, beta (Grp94), member1 (Hsp90b1)	0.765	0.924

ER stress-related gene expression levels obtained from the DNA microarray analysis are listed. The values are ratio to the control.

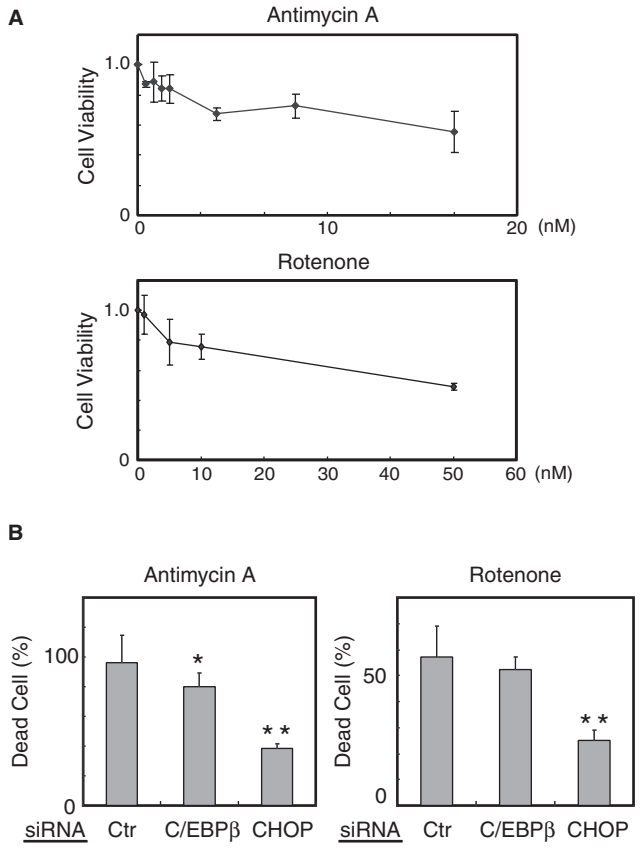

Fig. 6. **Attenuation of cytotoxicity by knockdown of CHOP during mitochondrial stress.** (A) NMuMG cells were grown in 96-well plates and incubated with rotenone and antimycin A at the indicated doses for 24 h. The cell viability was assessed by MTT assay as described in MATERIALS AND METHODS section, and the ratio of treated to the control (vehicle) is plotted. (B) C2C12 cells were transfected with siRNA duplexes (100 nM) with sequence #1 for CHOP and C/EBPβ. At 48 h post-transfection, cells were exposed to antimycin A (3.6 nM) and rotenone (20 nM), and cell viability was determined by the trypan blue exclusion assay. Dead cells stained with the dye were counted under a microscope, and the percentages are shown. The significance of the difference was assessed by *t*-test ($^{**}P < 0.005$ and $^{*}P < 0.05$).

during interference of the respiratory chain complex III by antimycin A reported a limited gene expression profile during adipogenesis with CREB identified as a key regulator (*12*).

In this study, two experimental models were examined in which either complex I or III was inhibited by rotenone (complex I) or antimycin A (complex III), respectively. Data contamination by irrelevant side-effects of chemicals was minimized by focusing on changes commonly observed in both treatments. Forty-eight genes were identified as *bona fide* candidates that responded to inhibition of the mitochondrial respiratory chain. This included the transcription factor CHOP, which is robustly expressed in response to a broad range of stress, in particular by ER stress. The induction of CHOP by mitochondrial stress was consistent with a previous report, in which induction was mediated by mitochondrial reactive oxygen species (*13*). It is noteworthy that distinctive responses were observed in a set of transcriptional regulators encoded by *E2F7*, *p107 and Zhx1* among the three cell lines, which might be responsible for the cell-type specificity of cellular responses during mitochondrial stress.

In most cases, the expression of CHOP in response to stress is regulated at the transcriptional level through the transcription factor ATF2, 4 and 6 (*9*). We observed the activation of p38 MAP kinase under treatment with antimycin A and rotenone (data not shown), which results in the activation of ATF2 in the nucleus. Therefore, together with the finding mentioned above (*13*) it is assumed that inhibition of the respiratory chain increases mitochondrial reactive oxygen species that activate p38 MAP kinase, thereby up-regulating CHOP through ATF2. In fact, the activation of p38 MAP kinase was involved in the induction of CHOP during stress signalling (*14*, *15*).

CHOP is a transcription factor with a well-established role in ER stress, particularly in the induction of apoptosis (*9*). Downstream targets are carbonic anhydrase VI (*16*), Bcl2 (*17*) and others (*18*). Moreover, CHOP has been associated with mitochondrial stress that was induced by accumulation of unfolded proteins (*19*). The targets in that particular situation were chaperonin 60, chaperonin 10, mtDnaJ and C1pP. In these cases, it was assumed that CHOP dimerized with C/EBPβ and regulated transcription of the target genes. In the current study, an important role of CHOP in response to mitochondrial stress was discovered (Figs 3, 4 and 6B). CHOP was shown to be involved in the regulation of *Atf3*, *Trib3 and Gsta1*. C/EBPβ was also involved in the up-regulation of *Trib3* and *Gsta1*. Thus, the up-regulation of these genes is most likely to be mediated by CHOP-C/EBPβ heterodimers, while the up-regulation of *Atf3* was possibly mediated by CHOP heterodimerizing with another partner. The involvement of other C/EBP family members such as C/EBPα, β and γ was possible,

although the result of the DNA microarray showed that unlike CHOP and C/EBPβ, they were not induced under mitochondrial stress (data not shown). With respect to the transcriptional regulators encoded by *E2F7*, *p107* and *Zhx1* that were down-regulated by mitochondrial stress in the epithelial and myoblastic cell lines, neither CHOP nor C/EBPβ were involved in the down-regulation (Fig. 3) with a responsible regulator remaining unidentified.

One of the downstream effector genes of interest is *Trib3*, an ER stress-inducible gene regulated by CHOP (*8*). In the current study, we found that *Trib3* was induced by mitochondrial stress and like ER stress, the induction was mediated by CHOP (Fig. 4). However, the underlying molecular mechanism was different between the two stress conditions. During ER stress, the signals acted via an ER stress response element identified in the promoter region of *Trib3*, which consisted of three identical tandem repeats containing a CHOP-binding site at the center (*8*); whereas mitochondrial stress did not activate transcription from this same element (Fig. 5). According to the report, CHOP partners with *Atf4* and regulates the transcription during ER stress-mediated induction of *Trib3* (*8*), while it cooperates with C/EBPβ during mitochondrial stress (Fig. 4). Thus, CHOP is likely to heterodimerize with distinct partners and regulates gene expressions in distinctive ways, depending upon the features of each individual stress, thereby coordinating individual transcriptional activities into adaptive cellular response as a whole adequate to a particular stress. Accordingly, the species and the relative amount of each binding partner of the CHOP heterodimer would provide a potential molecular framework that defines the transcriptional network and ultimately the cellular response.

We hope that the underlying regulatory cascades and gene expression patterns discovered in this study will aid in better understanding of the cellular responses during mitochondrial stress and development of therapeutic strategies for this stress, thereby contributing to the prevention and alleviation of toxicity caused by the mitochondria dysfunction accompanying such pharmacological treatments.

FUNDING

Grant-in-Aid for Scientific Research and the High-Technology Research Center Project from the Ministry for Education, Science, Sports and Culture of Japan; the High-Tech Research Center Project for Private Universities: a matching fund subsidy from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) 2004–2007.

CONFLICT OF INTEREST

None declared.

REFERENCES

1. Scatena, R., Bottoni, P., Botta, G., Martorana, G. E., and Giardina, B. (2007) The role of mitochondria in pharmacotoxicology: a reevaluation of an old, newly emerging topic. *Am. J Physiol. Cell. Physiol.* **293**, C12–C21
2. Kakkar, P. and Singh, B. K. (2007) Mitochondria: a hub of redox activities and cellular distress control. *Mol. Cell. Biochem.* **305**, 235–253
3. Fleischer, S. (1979) *Methods in Enzymology*, Vol. 53, Academic Press, New York
4. McComsey, G., Bai, R. K., Maa, J. F., Seekins, D., and Wong, L. J. (2005) Extensive investigations of mitochondrial DNA genome in treated HIV-infected subjects: beyond mitochondrial DNA depletion. *J. Acquir. Immune. Defic. Syndr.* **39**, 181–188
5. Lewis, W., Day, B. J., and Copeland, W. C. (2003) Mitochondrial toxicity of NRTI antiviral drugs: an integrated cellular perspective. *Nat. Rev. Drug. Discov.* **2**, 812–822
6. Kanome, T., Itoh, N., Ishikawa, F., Mori, K., Kim-Kaneyama, J. R., Nose, K., and Shibanuma, M. (2007) Characterization of Jumping translocation breakpoint (JTB) gene product isolated as a TGF-beta1-inducible clone involved in regulation of mitochondrial function, cell growth and cell death. *Oncogene* **26**, 5991–6001
7. Ishikawa, F., Nose, K., and Shibanuma, M. (2008) Downregulation of hepatocyte nuclear factor-4alpha and its role in regulation of gene expression by TGF-beta in mammary epithelial cells. *Exp. Cell Res.* **314**, 2131–2140
8. Ohoka, N., Yoshii, S., Hattori, T., Onozaki, K., and Hayashi, H. (2005) TRB3, a novel ER stress-inducible gene, is induced via ATF4-CHOP pathway and is involved in cell death. *EMBO J.* **24**, 1243–1255
9. Oyadomari, S. and Mori, M. (2004) Roles of CHOP/GADD153 in endoplasmic reticulum stress. *Cell Death Differ.* **11**, 381–389
10. Ubeda, M., Wang, X. Z., Zinszner, H., Wu, I., Habener, J. F., and Ron, D. (1996) Stress-induced binding of the transcriptional factor CHOP to a novel DNA control element. *Mol. Cell. Biol.* **16**, 1479–1489
11. Zinszner, H., Kuroda, M., Wang, X., Batchvarova, N., Lightfoot, R. T., Remotti, H., Stevens, J. L., and Ron, D. (1998) CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum. *Genes Dev.* **12**, 982–995
12. Vankoningsloo, S., De Pauw, A., Houbion, A., Tejerina, S., Demazy, C., de Longueville, F., Bertholet, V., Renard, P., Remacle, J., Holvoet, P., Raes, M., and Arnould, T. (2006) CREB activation induced by mitochondrial dysfunction triggers triglyceride accumulation in 3T3-L1 preadipocytes. *J. Cell Sci.* **119**, 1266–1282
13. Carriere, A., Carmona, M. C., Fernandez, Y., Rigoulet, M., Wenger, R. H., Penicaud, L., and Casteilla, L. (2004) Mitochondrial reactive oxygen species control the transcription factor CHOP-10/GADD153 and adipocyte differentiation: a mechanism for hypoxia-dependent effect. *J. Biol. Chem.* **279**, 40462–40469
14. Kultz, D., Madhany, S., and Burg, M. B. (1998) Hyperosmolality causes growth arrest of murine kidney cells. Induction of GADD45 and GADD153 by osmosensing via stress-activated protein kinase 2. *J. Biol. Chem.* **273**, 13645–13651
15. Oh-Hashi, K., Maruyama, W., and Isobe, K. (2001) Peroxynitrite induces GADD34, 45, and 153 VIA p38 MAPK in human neuroblastoma SH-SY5Y cells. *Free Radic. Biol. Med.* **30**, 213–221
16. Sok, J., Wang, X. Z., Batchvarova, N., Kuroda, M., Harding, H., and Ron, D. (1999) CHOP-Dependent stress-inducible expression of a novel form of carbonic anhydrase VI. *Mol. Cell. Biol.* **19**, 495–504
17. McCullough, K. D., Martindale, J. L., Klotz, L. O., Aw, T. Y., and Holbrook, N. J. (2001) Gadd153 sensitizes cells to endoplasmic reticulum stress by down-regulating Bcl2 and

perturbing the cellular redox state. *Mol. Cell. Biol.* **21**, 1249–1259

18. Wang, X. Z., Kuroda, M., Sok, J., Batchvarova, N., Kimmel, R., Chung, P., Zinszner, H., and Ron, D. (1998) Identification of novel stress-induced genes downstream of chop. *EMBO J.* **17**, 3619–3630
19. Zhao, Q., Wang, J., Levichkin, I. V., Stasinopoulos, S., Ryan, M. T., and Hoogenraad, N. J. (2002) A mitochondrial specific stress response in mammalian cells. *EMBO J.* **21**, 4411–4419