Free Radical Research, June 2011; 45(6): 672–680

informa
healthcare

Importance of mitochondrial dysfunction in oxidative stress response: A comparative study of gene expression profiles

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(Received date: 27 December 2010; Accepted date: 5 February 2011)

Abstract

Mitochondria are considered to play an important role in oxidative stress response since they are a source of reactive oxygen species and are also targeted by these species. This study examined the mitochondrial conditions in cells of epithelial origin that were exposed to H_2O_2 and found a decline in the membrane potential along with a specific loss of UQCRC1, a sub-unit of complex III, suggesting that mitochondrial dysfunction occurs upon exposure to oxidative stress. This observation led to the hypothesis that certain cellular responses to oxidative stress occurred because of mitochondrial dysfunction. When mitochondria-less (pseudo $\rho 0$) cells were examined as a model of mitochondrial dysfunction, striking similarities were found in their cellular responses compared with those found in cells exposed to oxidative stress, including changes in gene expression and gelatinolytic enzyme activities, thus suggesting that cellular responses to oxidative stress were partly mediated by mitochondrial dysfunction. This possibility was further validated by microarray analysis, which suggested that almost one-fourth of the cellular responses to oxidative stress were mediated by mitochondrial dysfunction that accompanies oxidative stress, thereby warranting a therapeutic strategy that targets mitochondria for the treatment of oxidative stress-associated diseases.

Keywords: *H_2O_2, pseudo $\rho 0$ cell, DNA microarray, MMPs, NMuMG cell*

Abbreviations: *COX5A, cytochrome c oxidase, sub-unit Va; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MMP, matrix metalloproteinase; mtDNA, mitochondrial DNA; ROS, reactive oxygen species; SDHA, succinate dehydrogenase complex, sub-unit A, flavoprotein (Fp); UQCRC1, ubiquinol-cytochrome c reductase core protein 1.*

Introduction

Oxidative stress is a common and fundamental cause of a wide range of degenerative diseases [1]. Under oxidative stress conditions, excessive reactive oxygen species (ROS) that are produced either enzymatically or non-enzymatically overwhelm the antioxidative capacities of target cells and deleteriously modify macromolecules such as lipids, proteins and DNA, thereby exhibiting pleiotropic effects on these cells, including epigenetic and genetic effects.

Mitochondria contain a cellular compartment that plays pivotal roles in oxidative stress [2–5]. This compartment is a major site of ROS production within cells where the respiratory chain consumes 90% of the total cellular oxygen and converts ~ 1–3% of molecular oxygen into superoxide radicals. Consequently, mitochondria are also prospective targets of ROS within cells. In fact, mitochondrial DNA (mtDNA) has been demonstrated to undergo more oxidative modification than nuclear DNA [2], possibly due to its proximity to the sites of ROS production and vulnerability to mutagenic lesions, which is attributable to its peculiar features such as the lack of histone protection and limited DNA repair capacity.

The damage in mtDNA in turn generally leads to direct and indirect loss of the components of the respiratory chain complexes, resulting in entire defects in the respiratory function as described below. Notably, the

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2011 Informa UK, Ltd.
DOI: 10.3109/10715762.2011.564169

mutations and deletions in mtDNA are fatal because mtDNA is devoid of introns and is exclusively coded to build multi-enzymatic machinery for oxidative phosphorylation, including rRNA and tRNA for the translation. Thus, lesions in mtDNA often result in direct loss or deficiency in components of the respiratory chain. Besides, it is possible that some of the nuclear-encoded components of the respiratory chain are also lost in an indirect manner as discussed by Marusich et al. [6]. Importantly, resultant dysfunction of the respiratory chain generally leads to leakage of electrons to oxygen or increased ROS production, which was defined in the cells exposed to inhibitors of the respiratory complexes, such as rotenone and antimycin A [5]. Accordingly, damage to mtDNA structurally and functionally disrupts the respiratory chain in most cases, leading to inappropriate generation of ROS and increased mtDNA damage, a consequence of which is the catastrophic cycle of respiratory function dysregulation that leads to further ROS generation. Eventually this cycle results in devastating cellular injury. In summary, oxidative stress and mitochondrial respiratory dysfunction occur at the same time to potentiate oxidative stress-associated morbidities.

Based on the above-mentioned factors, mitochondrial dysfunction has often been implicitly associated with oxidative stress in the literature. However, few studies have directly addressed mitochondrial dysfunction and its role in oxidative stress. In the case of myocardial failure, chronic increases in ROS production have been reported to impair mitochondrial function, although the significance of this phenomenon remains unexplored [7].

Previously, we studied the effects of long-term oxidative stress on epithelial cells and observed the remarkable morphological and phenotypical alterations induced by stress. The expression of genes such as matrix metalloproteinases (MMPs) and integrins was affected and, eventually, the cells acquired a motile phenotype [8]. In this study, we examined the possibility that such cellular changes resulted from mitochondrial dysfunction, at least partially, which was in turn caused by oxidative stress.

Materials and methods

Cell culture and mitochondria-less (pseudo ρ0) cell preparation

NMuMG mouse mammary epithelial cell line was obtained from the American Type Cell Collection (Manassas, VA) and maintained as described previously [9]. For the preparation of pseudo $\rho 0$ cells, the cells were cultured in a normal medium containing 250 ng/ml ethidium bromide (EtBr) and 50 μg/ml uridine for 4–7 days [10].

JC-1 and H_2DCF staining

Mitochondrial membrane potential ($\Delta\Psi m$) and intracellular ROS production were monitored as described previously using 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazole (JC-1) [9] and 2′,7′-dichlorofluorescein (H_2DCF) diacetate [11], respectively.

Real-time RT-PCR

Total RNA from cultured cells was extracted and cDNA was synthesized and analysed by methods described previously [12]. The primers were designed by Primer Express software v3.0 for real-time PCR (Applied Biosystems, Carlsbad, CA).

Antibodies and Western blotting

The procedure for Western blotting was essentially the same as that described previously [13]. Monoclonal antibodies used were anti-UQCRC1 (Invitrogen, Camarillo, CA), anti-SDHA (Invitrogen), anti-COX5A (Invitrogen), anti-prohibitin (Lab Vision Corporation, Fremont, CA), anti-cytochrome c (BD Biosciences, San Jose, CA) and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Chemicon, Temecula, CA). Anti-Mn-SOD polyclonal antibody was purchased from Upstate (Lake Placid, NY).

DNA microarray

DNA microarray analysis was performed according to the Two-Colour Microarray Gene Expression Analysis protocol as described previously [12]. Total RNA samples isolated from cells were reverse-transcribed and cyanine 3- or 5-labelled cRNA was synthesized. Then, equal amounts of cRNA samples per pair were hybridized to whole mouse genome (4×44k) Oligo Microarray chips (Agilent Technologies, Santa Clara, CA). Image, normalization and statistical analyses of primary spot intensity were performed with Feature Extraction Software (Agilent Technologies). Selection of genes was conducted with Gene Spring (Agilent Technologies).

Gelatin zymography

The procedure was essentially the same as that described previously [8]. Cells were incubated in serum-free medium for 24 h, after which the conditioned medium was collected and concentrated. After normalizing for cell number with the sample buffer, the medium was separated by electrophoresis on 7.5% polyacrylamide gels containing 1 mg/ml gelatin as a substrate.

Results

Mitochondrial dysfunction caused by oxidative stress

Mouse mammary epithelial NMuMG cells were exposed to H_2O_2 (0.2 mM) for 2–4 days, as previously described

[8], and the expression of several mitochondrial proteins was examined by Western blotting. The results show that the expression of ubiquinol-cytochrome c reductase core protein 1 (UQCRC1), a nuclear-encoded sub-unit of the complex III of the respiratory chain, was specifically decreased (Figure 1A). Complex IV sub-unit Va (COX5A; cytochrome c oxidase, sub-unit Va) and complex II 70kDa sub-unit (SDHA; succinate dehydrogenase complex, sub-unit A, flavoprotein (Fp)), which are also encoded in the nuclear DNA, showed a slight decrease (COX5A) or a marginal change (SDHA) in their expression levels.

Consistent with the observed decrease in the expression of UQCRC1, the activity of the respiratory chain, or $\Delta\Psi m$, which was monitored using the membrane potential-sensitive probe JC-1, significantly declined to levels that were less than half of those noted under normal conditions on day 4 of treatment (Figures 1B and C). Notably, UQCRC1 was recently identified as a protein that is most susceptible to mitochondrial stress [14]. Therefore, the exposure to oxidative stress was shown to have detrimental effects on mitochondria, which were manifested by decreases in the nuclear-encoded component of the respiratory chain and $\Delta\Psi m$. Mn-SOD, prohibitin and cytochrome c levels remained unchanged (Figure 1A); therefore, it appears that mitochondria-localized proteins were not randomly lost, indicating that the mitochondria were not severely damaged. Rather, the function of the respiratory chain appears to have been selectively disrupted.

As shown in Figure 1D, ROS levels in the cells treated with H_2O_2 were found to be elevated shortly after treatment. However, the steady-state levels of ROS declined after long-term treatment with oxidants (Figure 1E), possibly due to the resultant mitochondrial dysfunction as described above. In fact, ROS levels were decreased when the respiratory function was disrupted in pseudo $\rho 0$ cells, a model of mitochondrial dysfunction, as described below (Figure 2D).

A model of mitochondrial dysfunction

Based on the above observations, we postulated that cellular responses to oxidative stress essentially resulted, at least partially, from stresses to the mitochondria, thereby being regulated by mitochondrial stress signals rather than those directly activated by the oxidants. To test this possibility, we subjected cells directly to mitochondrial stress and tested whether certain cellular responses to oxidative stress were similarly induced under mitochondrial stress conditions. The model of mitochondrial stress condition employed in this study used the pseudo $\rho 0$ state that was attained by treating cells with EtBr, which inhibits mitochondrial gene transcription and replication, thereby depleting mtDNA [10].

As shown in Figure 2A, EtBr treatment significantly decreased the expression of mitochondria-encoded genes, a sub-unit of NADH hydrogenase (ND1), and cytochrome b after 4 days, thus ensuring that mitochondrial transcription was indeed inhibited. Expression levels were slightly restored after 6 days, possibly due to the induction of resistance to the agent, which has been previously reported in mouse cell lines [15]. Among the mitochondria-localized proteins shown in Figure 2B, the expression of UQCRC1 decreased dramatically, whereas that of other proteins remained essentially unchanged (the expression of COX5A was slightly reduced). This expression pattern was the same as that caused by oxidative stress (Figure 1A).

As a consequence of the decreased expression of mitochondrial proteins constituting the respiratory chain (Figures 2A and B), $\Delta\Psi m$ of the pseudo $\rho 0$ cells was markedly decreased (Figure 2C) and levels of intracellular ROS were reduced after 4 days (Figure 2D), although it recovered on day 7 concomitantly with the recovered expression of the above-mentioned mitochondria-encoded transcripts (Figure 2A). Of note, all the behaviours of the $\rho 0$ cells were also observed in the cells treated with oxidants (Figure 1), lending support to the relevance of the pseudo $\rho 0$ state as a model of mitochondrial dysfunction caused by oxidative stress conditions.

Given that dysfunction of the respiratory chain leads to increased ROS production [5], as mentioned in the Introduction, it was possible that the loss of some of the respiratory chain components as above caused disturbance of the respiratory chain activity in $\rho 0$ cells, resulting in ROS production. In fact, the ROS levels were apparently increased on day 0.5 and 1 after EtBr treatment. However, the increases were small and not statistically significant (Figure 2D).

Comparison of gene expression under oxidative and mitochondrial stress conditions

In a previous study, we found that repeated exposure to oxidative stress caused remarkable morphological changes that were accompanied by alterations in gene expression, including that of MMPs and integrins, in epithelial cells [8]. In the present study, we determined whether similar changes are observed under mitochondrial stress conditions, assuming that some of the responses to oxidative stress originated from mitochondrial stress occurring as a consequence of the oxidative stress. In fact, the responses were strikingly similar between the two stress conditions, including the induction of MMP-13 at the mRNA level (Figures 3A and B), a dramatic increase in the zymogen of MMP-2 and proteolytic activation of MMP-9 (Figure 3C) [8]. Taken together with changes observed in integrin sub-unit expression, which were also essentially the same between the two conditions (data not shown) [8], these comparative studies revealed a considerable concordance in gene expression patterns under the two stress conditions. Although further study should be needed to clarify cause and effect, it is plausible

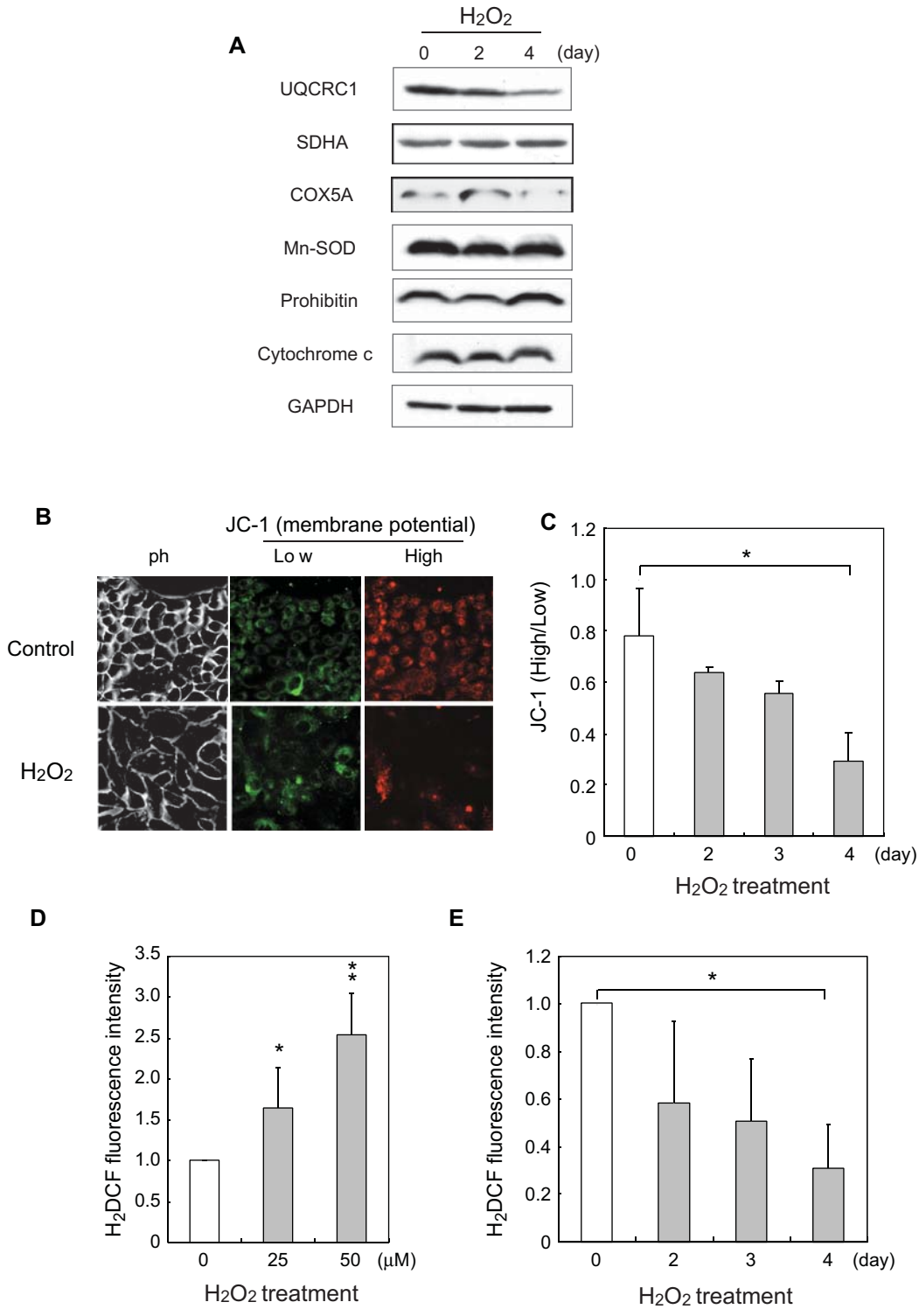

Figure 1. Mitochondria dysfunction caused by oxidative stress. NMuMG cells were treated with H_2O_2 (0.2 mM) for 4 days as previously described [8] and protein expression (A), ΔΨm (B, C) and H_2O_2 levels were examined (D). (A) Cell lysates were prepared on the indicated day and analysed by Western blotting with antibodies specific to each protein (Materials and methods). GAPDH was used as the loading control. (B, C) On each day of treatment, ΔΨm was examined by JC-1 staining (Materials and methods). Mitochondria with an intact ΔΨm (higher than 100 mV) concentrate JC-1, which fluoresces green as a monomer, into aggregates that fluoresce red. (B) Images taken on day 4 of treatment. Low and High, the corresponding images of green and red fluorescence of JC-1 captured on a fluorescence microscope; Ph, phase-contrast images. The fluorescence intensities of the images were analysed using Aquacosmos software (Hamamatsu Photonics, Hamamatsu, Japan) and the relative intensities to the control (day 0) are shown (C). Values are expressed as means of four independent samples ± SD. The significance of the differences was assessed using the *t*-test ($^{*}p < 0.05$). (D, E) ROS levels were measured using H_2DCF and the fluorescence images captured on the microscope were analysed using the software described in the Materials and methods section. In Figure 1D, the ROS levels were measured in the cells treated with H_2O_2 at the indicated doses for 10 min. Values are expressed as the means of three independent samples ± SD relative to the control. The significance of the differences was assessed by *t*-test ($^{*}p < 0.05$, $^{**}p < 0.005$).

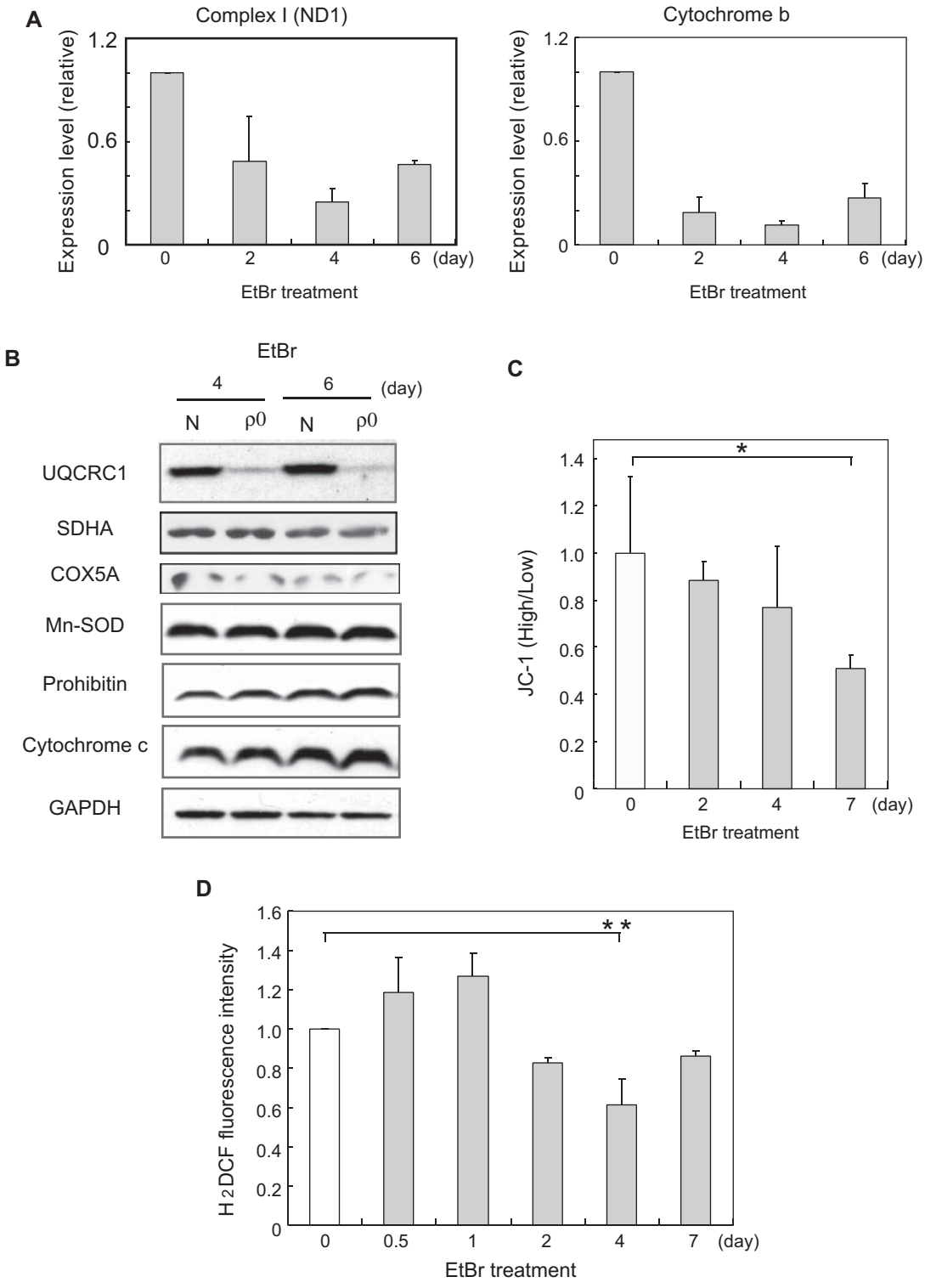

Figure 2. Pseudo $\rho 0$ cells derived from NMuMG cells. NMuMG cells were treated with EtBr (250 ng/ml) as described in Materials and methods section and examined for the expression of mitochondrial transcripts (A), mitochondrial protein levels (B), $\Delta\Psi m$ (C) and ROS production (D). (A) Total RNA was extracted and analysed by real-time RT-PCR using primers specific to the target genes. mRNA quantities were normalized with that of GAPDH and are shown relative to the normal cells. (B) Cell lysates prepared from the normal (N) and EtBr-treated ($\rho 0$) cells were examined by Western blotting as described in Figure 1A. (C) JC-1 staining and evaluation were performed as described in Figure 1C. Values are expressed as means of three independent samples ± SD. (D) ROS levels were measured using H_2DCF, as described above. Values are expressed as means of four independent samples ± SD relative to the control. The significance of the differences was assessed by *t*-test ($^{*}p < 0.05$, $^{**}p < 0.005$).

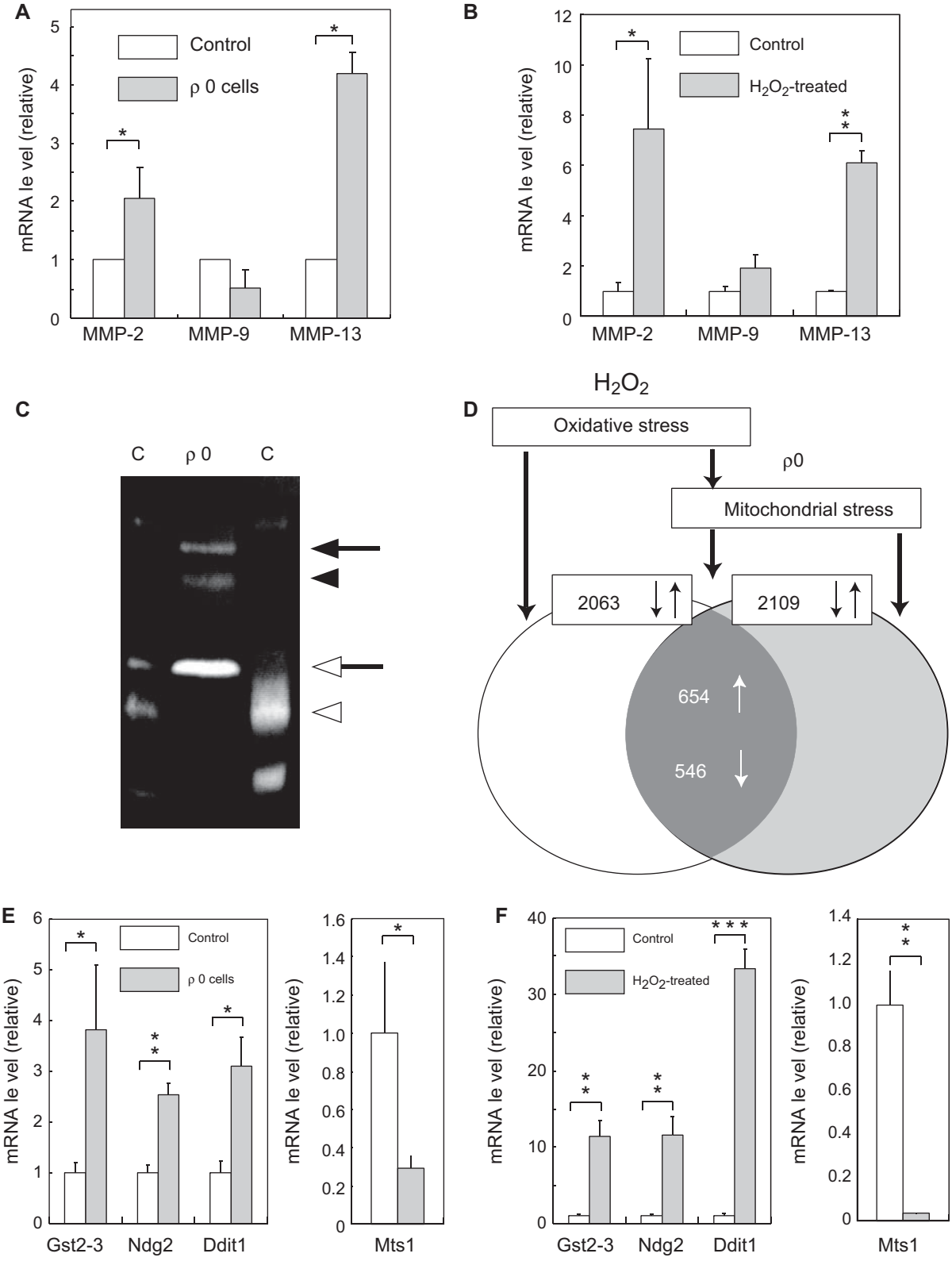

Figure 3. Changes in gene expression in pseudo $\rho 0$ and H_2O_2-treated cells. (A–C, E and F) Total RNA and conditioned medium were obtained from the control (C), pseudo $\rho 0$ (treated with EtBr for 4 days) (A, C, E) and H_2O_2-treated cells (treated with H_2O_2 [0.2 mM] for 4 days as described previously [8]) (B, F) and analysed by real-time RT-PCR (A, B, E and F) and gelatin zymography (C), respectively. The mRNA quantities are shown as in Figure 2A. Values are expressed as means of three independent samples ± SD. The significance of the differences was assessed by *t*-test ($^{*}p < 0.05$, $^{**}p < 0.005$, $^{****}p < 0.000\,05$). The arrows in (C) indicate pro-MMP-9 (solid) and -MMP-2 (empty) and the corresponding arrowheads indicate MMP-9 and MMP-2, respectively. (D) Summary of the microarray analysis performed for the changes in gene expression under oxidative and mitochondrial stress conditions (see Table I and text).

that certain oxidative stress responses are mediated by mitochondrial dysfunction.

Finally, we extended the above pilot study to include a genome-wide analysis of the gene expression that occurs under the two stress conditions, by performing DNA microarray analysis. cRNA pairs generated based on RNA obtained from the control and H_2O_2- or EtBr-treated cells were hybridized with a mouse DNA microarray for this analysis. As summarized in Figure 3D, ~ 2000 genes were up- or down-regulated by as much as 2-fold under each stress condition and, notably, approximately half of these changes were shared between the two conditions. The overlapping genes (the top 20 genes that were up- or down-regulated by H_2O_2) are listed in Table I. Real-time RT-PCR verified the up-regulated (Gsat2-3, Ndg2 and Ddit1) and down-regulated (Mts1) changes that were observed in cells treated with EtBr (Figure 3E) and H_2O_2 (Figure 3F).

Table I. Gene list of microarray analysis. Total RNA was extracted from normal (C), H_2O_2-treated (H), mitochondria-less (pseudo ρ0) (ρ) and H_2O_2-treated ρ0 (ρH) NMuMG cells. For each combination, C/H; normal vs H_2O_2-treated, C/ρ; normal vs ρ0, ρ/ρH; ρ0 vs H_2O_2-treated ρ0, three independent pairs of samples were generated and hybridized with a whole mouse genome microarray. Analysis was performed as described in the Materials and methods section. For selection of genes, first, genes that showed a change of more than 2-fold after H_2O_2- or EtBr-treatment were extracted. Then, among these genes, those overlapping between the two treatments were extracted and the top 20 genes (each of up- and down-regulation) are listed in the order of the fold changes after H_2O_2 treatment. The average listed is the mean of the fold changes (control vs H_2O_2-treated or ρ0) obtained from the three independent pairs for each combination.

		C/H		C/ρ		ρ/ρH	
Gene name	Official name	Average	SD	Average	SD	Average	SD
Up-regulation							
Gst2-3	glutathione S-transferase, alpha 3	37.03	40.69	22.65	23.41	2.60	1.18
Esg1	developmental pluripotency associated 5	25.51	15.42	5.96	4.15	6.53	4.67
sut; xCT	solute carrier family 7, member 11	24.88	18.62	6.23	4.51	4.66	1.43
Avdp	aldo-keto reductase family 1, member B7	23.98	17.87	26.10	26.38	1.74	0.72
COXVIaH	cytochrome c oxidase, sub-unit VI a	23.71	11.99	25.21	13.07	2.05	1.66
Gst2-1	glutathione S-transferase, alpha 1 (Ya)	22.05	19.21	5.51	2.27	4.76	1.30
DOC1	carbonic anhydrase 6	22.04	9.33	19.23	8.18	4.85	3.24
A_51_P305138		15.22	7.91	4.70	1.60	4.14	1.39
Ndg2	Nur77 downstream gene 2	15.20	4.69	14.17	5.56	1.83	0.94
MGC19039	aldehyde dehydrogenase 1 family, member L2	12.36	5.19	10.09	3.42	1.99	1.44
Gstc-2	glutathione S-transferase, alpha 2 (Yc2)	12.00	5.88	4.59	1.43	4.24	0.72
Out	transcription factor 23	10.90	7.04	2.45	1.00	3.04	0.94
Gm566	gene model 566 (NCBI)	10.45	6.85	1.92	0.79	3.80	1.49
Ddit1	growth arrest and DNA-damage-inducible 45 alpha	10.16	3.46	9.88	4.73	1.50	0.86
AI844617	dual specificity phosphatase 4	9.04	2.14	7.97	6.43	3.54	2.65
Nipk; TRB-3	tribbles homologue 3 (Drosophila)	9.03	2.72	7.32	2.18	1.44	0.57
1810008K03Rik	ChaC, cation transport regulator-like 1 (E. coli)	8.71	5.85	4.75	2.72	1.84	0.79
GTPCH	GTP cyclohydrolase 1	8.62	4.33	7.23	3.06	1.54	0.26
Gst2-3	glutathione S-transferase, alpha 3	8.62	5.54	8.10	6.51	2.08	0.81
Wnt-7a	wingless-related MMTV integration site 7A	8.06	1.43	7.95	3.56	1.58	1.22
Down-regulation							
Ki-67	antigen identified by monoclonal antibody Ki 67	0.05	0.04	0.18	0.04	0.17	0.13
Mts1	S100 calcium binding protein A4	0.05	0.01	0.07	0.02	0.35	0.13
TIAP	baculoviral IAP repeat-containing 5	0.06	0.04	0.18	0.05	0.21	0.17
mPAL	Shc SH2-domain binding protein 1	0.06	0.04	0.19	0.06	0.26	0.15
TOPK	PDZ binding kinase	0.06	0.04	0.25	0.04	0.20	0.16
Cox2	complete cds; mitochondrial gene for mitochondrial product	0.06	0.01	0.19	0.07	0.03	0.01
MFT.M05.13	RIKEN cDNA 5730507H05 gene	0.07	0.05	0.24	0.11	0.26	0.22
BE368005	NCI_CGAP_Lu29 Mus musculus cDNA clone	0.08	0.01	0.26	0.14	0.05	0.01
MPK38	maternal embryonic leucine zipper kinase	0.08	0.04	0.19	0.06	0.32	0.21
Cenp-A	centromere protein A	0.08	0.07	0.34	0.22	0.29	0.21
H1B; H1.5; H1f5	histone 1, H1b	0.08	0.04	0.14	0.03	0.47	0.03
Msa	exonuclease 1	0.09	0.04	0.15	0.01	0.38	0.17
Kns4	kinesin family member 4	0.09	0.07	0.34	0.20	0.24	0.22
Ccn1; Ccna	cyclin A2	0.09	0.06	0.31	0.12	0.18	0.15
BC020382	Mus musculus cDNA clone IMAGE:3582855	0.09	0.02	0.28	0.15	0.08	0.03
X57780	M.musculus mRNA for mitochondrial gene for sub-unit I of cytochrome c oxidase	0.09	0.01	0.13	0.06	0.07	0.03
Reca; Rad51a	RAD51 homologue (*S. cerevisiae*)	0.09	0.05	0.15	0.01	0.36	0.13
Np95	ubiquitin-like, containing PHD and RING finger domains, 1	0.09	0.05	0.17	0.05	0.34	0.15
galectin-4	lectin, galactose binding, soluble 4	0.09	0.01	0.31	0.12	0.32	0.10
AV109292	spindle pole body component 24 homologue (*S. cerevisiae*)	0.09	0.06	0.19	0.02	0.29	0.12

Standard deviation is indicated as SD. *p*-values assessed by *t*-test were between 0.06–0.07. The candidate genes are in italics (see text).

It could be argued that the observed concurrence was fortuitous in some cases, being induced independently under these two conditions. In an attempt to discriminate this as a casual coincidence, we performed additional profiling of genes responding to oxidative stress in pseudo $\rho 0$ cells instead of normal cells. If a response was mediated by mitochondrial stress, the response to oxidative stress in this case was expected to be alleviated because in pseudo $\rho 0$ cells any response to oxidative stress was assumed to be induced earlier by mitochondrial stress signals. As shown in the third column, many of the responses (ρ/ρH) were affected in an expected manner and reduced compared with C/H (Table I).

The genes that almost equally responded to either stress (C/H and C/ρ) and whose responses to oxidative stress were affected in the $\rho 0$ condition by more than 2-fold (ρ/ρH) were underlined as prospective candidates that are possibly and primarily regulated by mitochondrial stress signals triggered by oxidative stress conditions. Thus, it is likely that, in total, approximately one-fourth of the responses to oxidative stress were potentially mediated by mitochondrial stress.

Discussion

Oxidative stress has been implicated in the pathogenesis of numerous diseases, including atherosclerosis, cancer, diabetes, ischemia/reoxygenation injury and alcohol- and drug-induced toxicity [1]. For the development of better or novel therapeutics and preventive strategies for these conditions, a detailed understanding of signals and effectors governing cellular responses to stress is essential. Clarifying key factors and, if possible, quantitatively evaluating the contribution of each factor to morbidity are also important for finding the appropriate molecular targets for new therapeutics.

In this study, we attempted to validate the currently prevailing view that mitochondrial dysfunction underlies oxidative stress and also to estimate the proportion of oxidative stress responses that are mediated by mitochondrial dysfunction, thereby warranting a strategy that targets mitochondria in order to treat the above-mentioned conditions. In isolated mitochondria as well as vascular endothelial and smooth muscle cells, mitochondrial dysfunction has been shown to occur under oxidative stress conditions, although this was only evaluated after a short-term insult within an hour [7,16]. In this study, we treated cells of epithelial origin with an oxidant in a repetitive manner, mimicking chronic inflammatory conditions that occur *in vivo* by which most degenerative diseases are caused, and studied the long-term cumulative effects of oxidative stress on mitochondria. Features of mitochondrial dysfunction, similar to those described in previous studies, were observed in the epithelial cells (Figure 1) and were also suggested to have an intermediary role in cellular responses elicited by oxidative stress. Notably, cellular alterations in gene expression and gelatinolytic enzyme activities that have been previously described following repetitive exposure to oxidative stress [8] were observed in pseudo $\rho 0$ cells (Figures 3A and C), which served as the model of mitochondrial dysfunction that accompanies oxidative stress in this study. The similarities in cellular alterations that were observed in these two stress conditions lend support to the role of mitochondrial dysfunction in the induction of cellular responses to oxidative stress. It might be argued that mitochondrial dysfunction due to oxidative stress is not necessarily equivalent to that observed in the $\rho 0$ state. However, it is believed that like the $\rho 0$ state, mitochondria eventually lose their functions entirely after exposure to oxidative stress conditions because of the vicious cycle from ROS production to mtDNA damage. In fact, similarly impaired features, including reduced $\Delta\Psi$m and specific loss of UQCRC1 (Figure 1 vs Figures 2B and C), occurred under both oxidative stress and $\rho 0$ conditions, supporting the relevance of the pseudo $\rho 0$ state as a model of oxidative stress-induced mitochondrial dysfunction. The above-mentioned oxidative stress-induced cellular alterations observed in the pseudo $\rho 0$ cells also validate this assumption.

Among the nuclear-encoded components of the respiratory chain—UQCRC1, SDHA and COX5A—the protein expression levels of SDHA and COX5A were in sharp contrast to that of UQCRC1 under mitochondrial stress conditions. SDHA levels remained unchanged and that of COX5A levels were slightly decreased. These differential steady-state expression levels of these nuclear-encoded components of the respiratory chain were presumably due to, at least in part, differences in the protein turnover rates under stress conditions, which were affected by protein–protein interactions, a partial assembly of the complex and/or transcriptional regulation as discussed by Marusich et al. [6]. Alternatively, it might be argued that chromosome regions containing genes such as *UQCRC1* but not *SDHA* are specifically susceptible to mutagenic lesions and selectively modified or damaged in the nucleus by oxidants and EtBr. To exclude this possibility, the analysis of cells lacking mitochondria by another method would be helpful.

Importantly, the results of the microarray analysis suggested that, after oxidative stress, almost one-fourth of gene expression changes were downstream of mitochondrial stress signalling, thus providing a quantitative estimate of the contribution of mitochondrial dysfunction to oxidative stress responses for the first time. Thus, this study reinforces the notion that mitochondria are a promising target for therapeutics designed to treat degenerative diseases that are triggered by oxidative stress and highlights the importance of protecting mitochondria from oxidative damage. Theoretically antioxidants that target mitochondria are ideal agents to prevent the vicious cycle of ROS production and mitochondrial damage and could be used to mitigate

oxidative stress as a whole. In fact, MitoQ is a mitochondria-targeted antioxidant that has shown promising activity in phase II clinical trials for treating Parkinson's disease and liver damage associated with hepatitis C [17]. Other antioxidant drugs or dietary supplements that do not specifically target mitochondria are also currently being marketed or evaluated in clinical trials as potential therapeutic agents [17].

Alternatively, the study of mitochondrial stress signalling potentially provides other avenues for treating oxidative stress-based diseases. We recently studied the transcriptional network operating under mitochondrial stress conditions elicited by pharmacological inhibition of the respiratory chain and found that the CHOP-10 transcription factor plays a role in the regulation of gene expression [12]. In the $\rho 0$ state, CHOP-10 appeared to play a similar role and was found to be involved in the up-regulation of MMP-13 (data not shown). Hence, the transcription factor and/or the signalling pathways that activate it are potential alternative candidates that serve as the targets of therapeutic agents. Future studies on the mediators and pathways of stress response could possibly lead to the development of new medications that manipulate or intervene in the cellular responses to stress and, thus, ameliorate the effects of these diseases with a single agent or in conjunction with antioxidants.

Conclusion

We found that the oxidative stress did impair respiratory activity and mitochondrial stress induced responses similar to those induced by oxidative stress. Moreover, a comparative genome-wide DNA microarray analysis allowed the estimation of the contribution of mitochondrial dysfunction to cellular responses following oxidative stress. Notably, approximately one-fourth of the responses to the oxidative stress were potentially mitochondrial stress mediated.

Declaration of interest

This work was supported in part by a Grant-in-Aid for Scientific Research (C) (21570205) and the High-Technology Research Center Project from the Ministry for Education, Culture, Sports, Science and Technology (MEXT) of Japan and also by the High-Tech Research Centre Project for Private Universities: a matching fund subsidy from MEXT, 2007–2009.

This paper was first published online on Early Online on 18 March 2011.

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