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Research paper

Distinctive adaptive response to repeated exposure to hydrogen peroxide associated with upregulation of DNA repair genes and cell cycle arrest

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ABSTRACT

Many environmental and physiological stresses are chronic. Thus, cells are constantly exposed to diverse types of genotoxic insults that challenge genome stability, including those that induce oxidative DNA damage. However, most *in vitro* studies that model cellular response to oxidative stressors employ short exposures and/or acute stress models. In this study, we tested the hypothesis that chronic and repeated exposure to a micromolar concentration of hydrogen peroxide (H_2O_2) could activate DNA damage responses, resulting in cellular adaptations. For this purpose, we developed an *in vitro* model in which we incubated mouse myoblast cells with a steady concentration of ~50 μM H_2O_2 for one hour daily for seven days, followed by a final challenge of a 10 or 20X higher dose of H_2O_2 (0.5 or 1 mM). We report that intermittent long-term exposure to this oxidative stimulus nearly eliminated cell toxicity and significantly decreased genotoxicity (in particular, a > 5-fold decreased in double-strand breaks) resulting from subsequent acute exposure to oxidative stress. This protection was associated with cell cycle arrest in G2/M and induction of expression of nine DNA repair genes. Together, this evidence supports an adaptive response to chronic, low-level oxidative stress that results in genomic protection and up-regulated maintenance of cellular homeostasis.

1. Introduction

Oxidative toxicity is a frequent challenge to cellular homeostasis that can be triggered by a variety of endogenous and environmental factors. Molecular damage by oxidants impairs cellular viability and is associated with the development of several human diseases [1]. Therefore, the pathways that regulate reactive oxygen species (ROS) homeostasis are crucial to mitigate ROS toxicity. In fact, highly specific mechanisms have evolved that form the basis of oxidant scavenging and ROS signaling systems [2].

The effects of the excessive production of ROS, or the insufficiency of antioxidant defenses to neutralize them, are referred as oxidative stress [3–7]. These modifications can contribute to cellular dysfunction and, over time, to the development of complex pathologies like neurodegenerative diseases [8–10], cancer [11–13], hypertension, diabetes [14], and premature aging [6,15,16]. Reactive oxygen species can cause DNA damage, which is associated with progressive physiological dysfunction, diseases, and even mortality [1,17,18]. The cellular response to either acute (single high dose) or chronic (repeated low/moderate doses) exposure to oxidant agents is different. Acute exposure triggers a series of intracellular antioxidant defense mechanisms that counteract the damage caused; if these are not sufficient, cells will die by apoptosis or necrosis. However, if cells survive, exposure also results in upregulation of many antioxidant defenses. Therefore, in cells exposed chronically to sublethal stress, a series of adaptive responses occurs that may prevent or reduce damage and death *via* activation of cellular and molecular pathways that enhance the ability of the cell or organism to withstand more severe stress [4,19,20].

Hydrogen peroxide (H_2O_2) plays multiple roles in cells. At low concentrations, it is an essential oxygen metabolite and serves as messenger in cellular signaling pathways that are necessary for the growth, development and fitness of living organisms [21–23]. However, the involvement of H_2O_2 in numerous types of cell and

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tissue injuries is well documented [10,24], in particular at higher concentrations. Although H_2O_2 itself has low reactivity toward cell constituents, it is of great physiological importance because its uncharged and relatively unreactive nature allows it to diffuse to sites throughout the cell, where it is capable of forming potent ROS in the presence of trace amounts of metal ions [2,25]. Under physiological conditions, cells can protect themselves *via* H_2O_2-degrading enzymes that include glutathione peroxidases, catalase and peroxiredoxins [21,26,27]; the existence and evolutionary conservation of these defense systems demonstrates the importance of H_2O_2 toxicity. However, under pathological conditions, including acute oxidative stress, these cellular defenses can be overwhelmed, for example by increased levels of H_2O_2 [19,28]. Therefore, the study of mechanisms underlying adaptive responses to oxidative damage induced by H_2O_2 should provide understanding about the promotion and progression of ROS-related disorders, as well as how to protect cells and tissues against oxidative damage.

Most published studies of adaptation to *in vitro* oxidative stress have been done with short exposures and/or acute stress models that do not permit full induction of cellular processes that may result in an adaptive or hormetic response, because they employed a single dose of oxidants and short time lapses ($<$ 1day) [29–32]. Recently, however, some reports have used long-term continuous exposure protocols resulting in interesting cellular phenotypic changes associated with adaptive processes, including induction of antioxidant scavenging systems [33,34]. We sought to deepen our understanding of the cellular response to chronic oxidative stress by examining the impacts of such stress on DNA, one of the key macromolecular targets of oxidative damage. To this end, we set up a myoblast-derived cell culture-based model to study DNA damage responses and cellular adaptations to repeated exposure to subtoxic concentrations of hydrogen peroxide. We show that this regimen induced functional cellular changes that counteracted the subsequent acute exposure to oxidative stress, evidenced by decreased levels of cytotoxicity and genotoxicity in conjunction with cell cycle arrest in G2/M and induction of expression of nine DNA repair genes, suggesting concerted genomic protection and up-regulated maintenance of cellular homeostasis.

2. Materials and methods

2.1. Cell culture

Mouse-derived myoblast C2C12 cells (ATCC, CRL-1772) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS, Sigma), 100 μg/ml penicillin and 100 μg/ml streptomycin, in a humidified atmosphere of 5% CO_2/95% air at 37 °C. Exponentially growing cultures were used in all experiments. Cells were disaggregated by using trypsin-EDTA (0.1%-0.25 mM) for 2 min and then detached by a gentle mechanical blow; trypsin was inactivated with addition of 5 ml of complete medium. Cell suspensions were centrifuged at 700 g for 5 min, supernatant discarded and cell pellets resuspended in complete medium to a final concentration of 1.5×10^6 cells/ml suspension. Cell cultures were periodically subcultured before reaching monolayer confluence.

2.2. Treatment outline

To induce an adaptive response, cells were grown for seven days with a single split on the morning of the fourth day, following this outline: during the log phase of growth, pretreatments with chronic pro-oxidant dose were performed by a daily 1 h addition of 5 mU/ml of glucose oxidase (GlucOx, Sigma G6641) in serum-free medium to generate hydrogen peroxide to achieve a final concentration of ~50 μM H_2O_2 during that time lapse (see Supplementary File 1). Afterward, the GlucOx-containing medium was aspirated, cells were washed twice with PBS, and then replenished with pre-conditioned culture medium. On the last day of this process, cultures that had or had not undergone chronic pro-oxidant treatments were challenged with an acute dose of 0.5 or 1 mM H_2O_2 for 30 min and subsequently processed for different tests.

2.3. Determination of cell death

2.3.1. MTT assay

Cells (2×10^4 per well) were seeded into 96-well plates and incubated at 37 °C. After 24 h, 10 μl of a stock solution of MTT (Sigma M2128) at 5 mg/ml in PBS was added to each well (MTT final concentration of 0.5 mg/ml) and cells were incubated for 4 h in the dark at 37 °C. Then, 100 μl of acid isopropanol was added and the absorbance of each sample was measured by using an ELISA reader at 570 nm and expressed as the percentage of control.

2.3.2. Evaluation of mitochondrial membrane potential and cytoplasmic membrane integrity

To determine variations of mitochondrial membrane potential and cytoplasmic membrane damage, C2C12 cells were assayed by using DiOC6 (Molecular Probes D273) and propidium iodide (PI) (Sigma P4170). Cells were suspended in 500 μl of PBS containing 5 μl of 10 μM DiOC6 and 5 μl of 1 mg/ml PI, and then cell suspensions were incubated in the dark at room temperature during 20 min, washed, resuspended in PBS and analyzed by flow cytometry (Coulter EPICS XL).

2.4. ROS detection

Relative levels of ROS were measured using DCFDA (Sigma C6827). Cells were exposed to 0.1 mM DCFDA for 15 min after treatments, incubated at 37 °C, washed with PBS three times, and analyzed using a Coulter EPICS XL flow cytometer. Results were expressed as fold changes relative to untreated controls.

2.5. DNA damage quantification

2.5.1. Alkaline comet assay

Cell samples were diluted with PBS, and then 20 μl of cell suspension were mixed with 80 μl of 0.4% low melting point agarose (Sigma A9414) at 37 °C, layered onto a glass slide and placed overnight in cold lysis solution containing a solution of 2.5 M NaCl, 100 mM Na_2 EDTA, 10 mM Tris, 10% DMSO, and 1% Triton X-100. After rinsing twice in PBS, slides were treated for 20 min with a cold alkaline mixture of 300 mM NaOH and 1 mM Na_2 EDTA, pH $>$ 13, and then samples were analyzed by electrophoresis for 20 min at 300 mA. After electrophoresis, slides were neutralized and later stained with 0.01 mg/ml ethidium bromide; 50 cells were scored per sample. Each experiment was repeated twice with internal duplicates for each treatment. The DNA damage/nucleus was quantified using the endpoint measurement of % tail DNA by the Comet Assay Software Project (CASP) program.

2.5.2. Chromosome alterations assay

After treatments, cells cultured in T25 vessels were exposed to colcemid (0.1 μg/ml; Sigma, D1925) for 2 h. Chromosome preparations were made following standard procedures. After harvesting, cells were hypotonically shocked with a 2:1 mixture of Na-citrate (0.7%): KCl (0.56%), then fixed in methanol: acetic acid (3:1), spread onto water-chilled glass slides and stained with Giemsa (4%, pH 6.8, 15 min). Chromosome analysis was performed on coded slides. All types of unstable abnormalities were scored, namely chromatid

breaks, isochromatid breaks, acentric fragments, dicentrics, rings, multiradial figures, and shattered chromosomes. In parallel, the frequency of cells with alterations was also determined. A total of one hundred metaphases per sample per experiment were analyzed. Two independent experiments were carried out.

2.5.3. Extra long quantitative PCR

Genomic DNA was extracted and quantified using an automated extraction procedure, and DNA damage analysis was performed using extra-long quantitative polymerase chain reaction (XL-QPCR) as described by Furda et al. [35]. At least two time-separated QPCR reactions were performed on each sample and at least three biological replicates were analyzed per treatment and time point.

2.6. Cell cycle analyses

Cell samples were fixed in 70% ethanol and subsequently incubated with 100 µg/ml RNase (Sigma, R5000), stained with 100 µg/ml propidium iodide (Sigma, P4170) for 30 min, and analyzed for PI fluorescence using an EPICS XL flow cytometer. Percentages of cells in each phase were calculated using FlowJo.

2.7. DNA repair gene expression PCR-based array

To assess the transcriptomic effects of long-term exposure to oxidative stimuli in C2C12 cells, qRT-PCR-based microarray analyses were performed in both control cells and cells exposed to chronic GlucOx treatments. Briefly, total RNA was extracted using the Qiagen RNeasy Mini Kit, and first-strand cDNA was synthesized by using 250 ng total RNA from each sample and oligo-dT primed reverse transcription (RT^2 first strand kit, QIAGEN 330401), according to the manufacturer's instructions. Real-time qPCR was performed on Mouse DNA Repair RT^2 Profiler PCR Array (QIAGEN PAMM-042Z), which targets 84 core DNA repair-related genes. Gene arrays were processed according to the manufacturer's instructions. First, RT^2 SYBR Green/fluorescein qPCR Master Mix (QIAGEN 330503) was mixed with cDNA products. Then 25 µl of the aliquot mixture was loaded onto each well of a 96-well array. Real-time qPCR on the array was performed by using the BioRad C1000 thermal cycler, CFX96™ Real-time PCR detection system. The reaction was run under the following conditions: 10 min at 95 °C to activate Hot Start DNA polymerase, followed by 40 cycles of 15 s at 95 °C for denaturation, and 60 s at 60 °C for annealing (fluorescence detection). Relative changes in gene expression were calculated by the ΔCt method with adjustment for the average expression of housekeeping genes (*Actb, B2m, Gapdh, Gusb and Hsp90ab1)*. The data were analyzed with SABiosciences PCR array data analysis software.

2.8. Statistical analysis

Unless stated to the contrary, all data presented in this report represent results obtained from three independent experiments per treatment group, each done in duplicate or triplicate. Error bars represent standard errors of the mean (SEM). Analyses of variance (ANOVA) were performed *via* GraphPad Prism or Statview software, with *post-hoc* comparisons carried out by Fisher's protected least significant difference (FPLSD) tests. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. Chronic H_2O_2 treatment protected cells from subsequent H_2O_2-induced cytotoxicity and intracellular ROS accumulation

The cytotoxicity of the challenge dose of H_2O_2 was significantly less when cells were pretreated with GlucOx, *i.e.*, the generator of a chronic dose of H_2O_2 ($p < 0.0001$) (Fig. 1A), although both chronic and acute doses decreased cell viability on their own with respect to untreated control cells ($p < 0.0001$, FPLSD). Acute exposure to H_2O_2 increased mitochondrial transmembrane potential in naïve cells but not cells which had undergone chronic pro-oxidant exposure (Fig. 1B; $p = 0.0292$, interaction of H_2O_2 and pro-oxidant treatments). On the other hand, the acute challenge dose induced increased PI fluorescence, indicative of permeabilization of the cytoplasmic membrane associated with necrotic cell death, more without than with pre-treatment (Fig. 1C, $p = 0.0574$ interaction of H_2O_2 and pro-oxidant treatments).

To test whether pre-treatment would increase ROS scavenging or reduce ROS production, we measured the intracellular conversion of the non-fluorescent DCFDA dye to the highly fluorescent dichlorofluorescein, which results from exposure to ROS, particularly H_2O_2. The pre-treated cells displayed only a slight increase in fluorescence after H_2O_2 challenge, illustrating the clear protective effect of preconditioning with low concentrations of H_2O_2 (Fig. 1D). The acute challenge (1 mM H_2O_2) resulted in a significant ($p = 0.01$, FPLSD) and large (> 40-fold) increase in intracellular ROS. In comparison, chronic pro-oxidant treatment resulted in a slight (1.2-fold) increase with respect to untreated controls ($p = 0.02$, FPLSD). Thus, the protective effect was clear, resulting in diminished concentrations of ROS after H_2O_2 exposure ($p = 0.006$ for interaction of H_2O_2 and pro-oxidant treatments).

3.2. Chronic H_2O_2 exposure protected against subsequent H_2O_2-induced DNA damage

These results demonstrated that we had successfully developed a model of adaptation to repeated H_2O_2 exposure. Therefore, we next tested our primary question, whether pre-treatment would result in protection of DNA, a critical target of oxidative damage in the cell. Fig. 2A and B show the measurement of DNA strand breaks (mainly Single Strand Breaks, SSB) as percentage of tail DNA. The challenged cells, as expected, displayed a significant increase of tail DNA to 35.07%, as compared to 1.80% in untreated controls ($p < 0.001$; main effect of H_2O_2). When cells were chronically pretreated and then acutely challenged with 1 mM H_2O_2, DNA damage was reduced by 24.7% ($p = 0.0360$ by posthoc analysis) in comparison to the non-adapted and challenged cells, again indicating the protective effect of the pretreatment with low concentrations of H_2O_2. The average of tail DNA/nucleus in 50 µM H_2O_2 pretreated cells was statistically indistinguishable from the basal level of untreated cultures ($p = 0.9566$ by posthoc analysis).

SSBs are an important and common form of DNA damage, but double strand breaks (DSBs) are far more cytotoxic [4,36,37]. We next assessed DSBs by evaluating chromosomal abnormalities after chronic and acute oxidative treatments. Acute treatment induced an increase so large that it was difficult to quantify in the frequency of total chromosomal abnormalities per cell; for that reason, we decided to report chromosomal damage based on the percentage of damaged cells. We note that this is a conservative choice that likely underreports the damage, and therefore the protective effect that we measured. Results of clastogenicity tests are summarized in Fig. 2C and D. When C2C12 cells were challenged with 1 mM H_2O_2, we observed a large increase in damaged cells ($p < 0.0001$, FPLSD); this treatment induced a striking pattern of damage referred to as shattered chromosomes. The chronic oxidative treatment resulted in a significant decrease in the percentage of damaged cells, marked by a lack of shattered chromosomes ($p < 0.0001$ for H_2O_2 *vs.* pre-treatment). In cells chronically treated with low-level H_2O_2 (≈ 50 µM), abnormality frequencies were similar to those of control cells ($p = 0.43$, FPLSD). One explanation for the decreased DNA damage measured in pre-treated

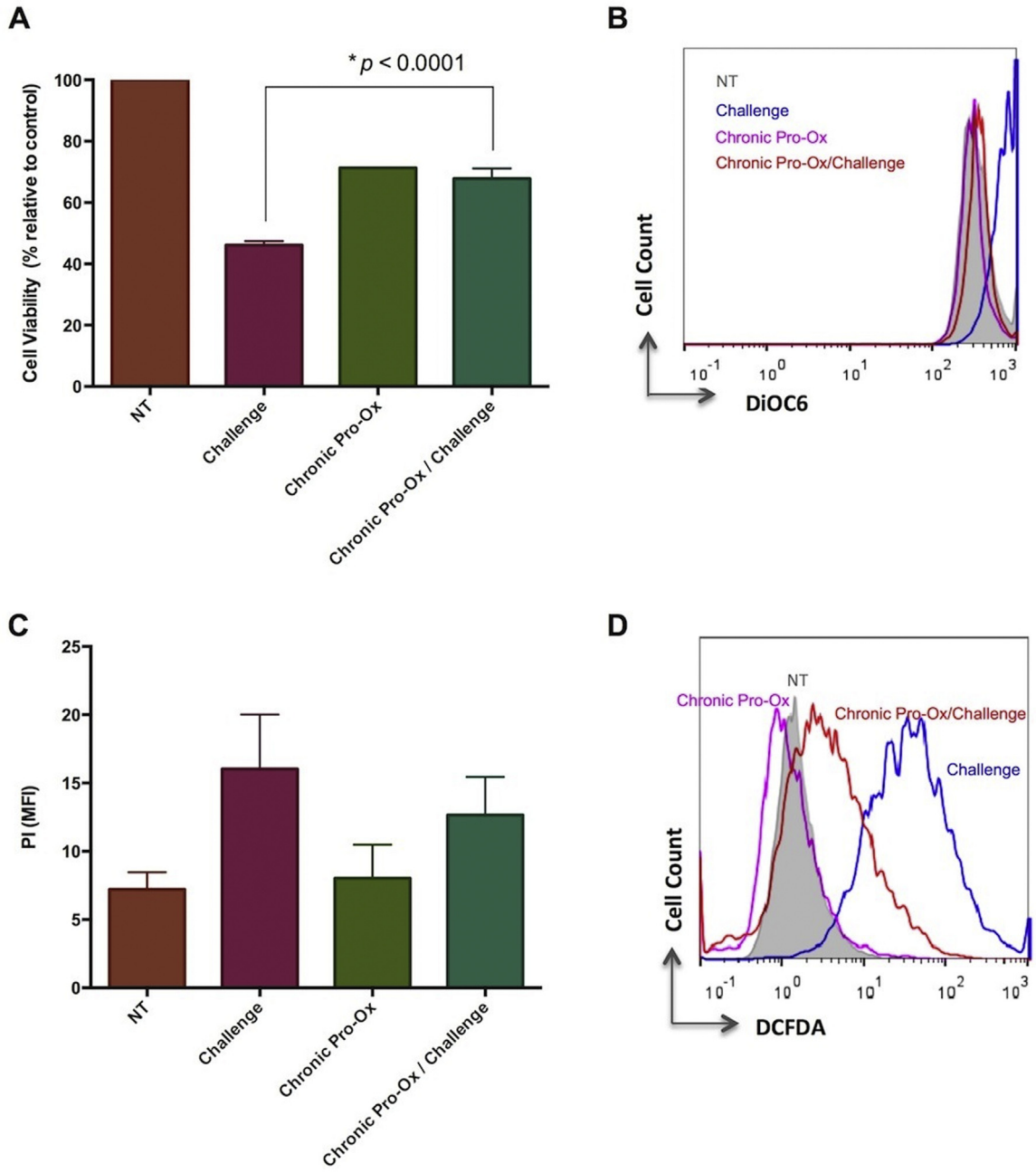

Fig. 1. Evidence of cytoprotection by chronic H_2O_2 pretreatments. C2C12 cells were treated under different pro-oxidant protocols. Cell viability was determined by (A) the MTT method; (B) DiOC6 uptake; and (C) PI incorporation. Values are expressed as mean ± SEM. Additionally, intracellular ROS was detected by flow cytometry; (D) shows a representative histogram for the increase in fluorescent intensity of DCF-DA produced by ROS in challenged cells.

cells is increased DNA repair, which we investigated next.

In addition, we extended some of our experimental procedures to a non-muscle derived cell line (CHO-K1 cells). As shown in Supplementary File 2, the results agreed with those reported for C2C12 cells.

3.3. Chronic H_2O_2 exposure induced expression of genes associated with DNA damage response and cell cycle arrest

To test for an induction of DNA repair in pre-treated cells, we first measured the expression levels of 84 genes involved in DNA Repair and compared to untreated cells. Among the 84 genes tested, nine genes *(Ape1, Atr, Ercc1, Mlh1, Msh6, Ogg1, Parp3, Rad18, Xrcc1)* were significantly up-regulated in chronic oxidative pretreated cells ($p < 0.05$ for > 1.5-fold change; Table 1), while significantly down-regulated genes were not detected. Supplementary File 3 shows the differential expression profile of those genes that did not show significant changes relative to non-treated (control) cells. The up-regulated genes are associated with cell cycle regulation, as well as different DNA damage response pathways; therefore, the up-regulation of these genes in pretreated cells is consistent with the hypothesis that chronic exposure to mild concentrations of H_2O_2 would activate DNA damage repair, cell cycle arrest, or both. Therefore, we next tested these possibilities.

3.4. Chronic H_2O_2 exposure had little or no impact on DNA repair kinetics

We measured H_2O_2 treatment-induced DNA lesions in nuclear and mitochondrial DNA at 0, 2, 6, and 24 h post challenge. nDNA damage was 0.19 lesions/10 kb after H_2O_2 exposure in pre-treated

cells, *i.e.*, lower than the 0.29 lesions/10 kb measured after the challenge treatment (Fig. 3A), but the trend toward faster nDNA repair was not statistically significant (two-way ANOVA; Fisher's PLSD, $p=0.2602$ for 0 h, $p=0.1794$ for 2 h, $p=0.2924$ for 6 h and $p=0.4719$ for 24 h). Likewise, mtDNA damage was lower in pretreated cells, and repair kinetics were not detectably altered (two-way ANOVA; Fisher's PLSD, $p=0.1179$ for 0 h, $p=0.2847$ for 2 h, $p=0.5853$ for 6 h and $p=0.6397$ for 24 h). As expected, mtDNA accumulated more lesions than did nuclear DNA [38,39], with damage levels in the mitochondrial genome approximately

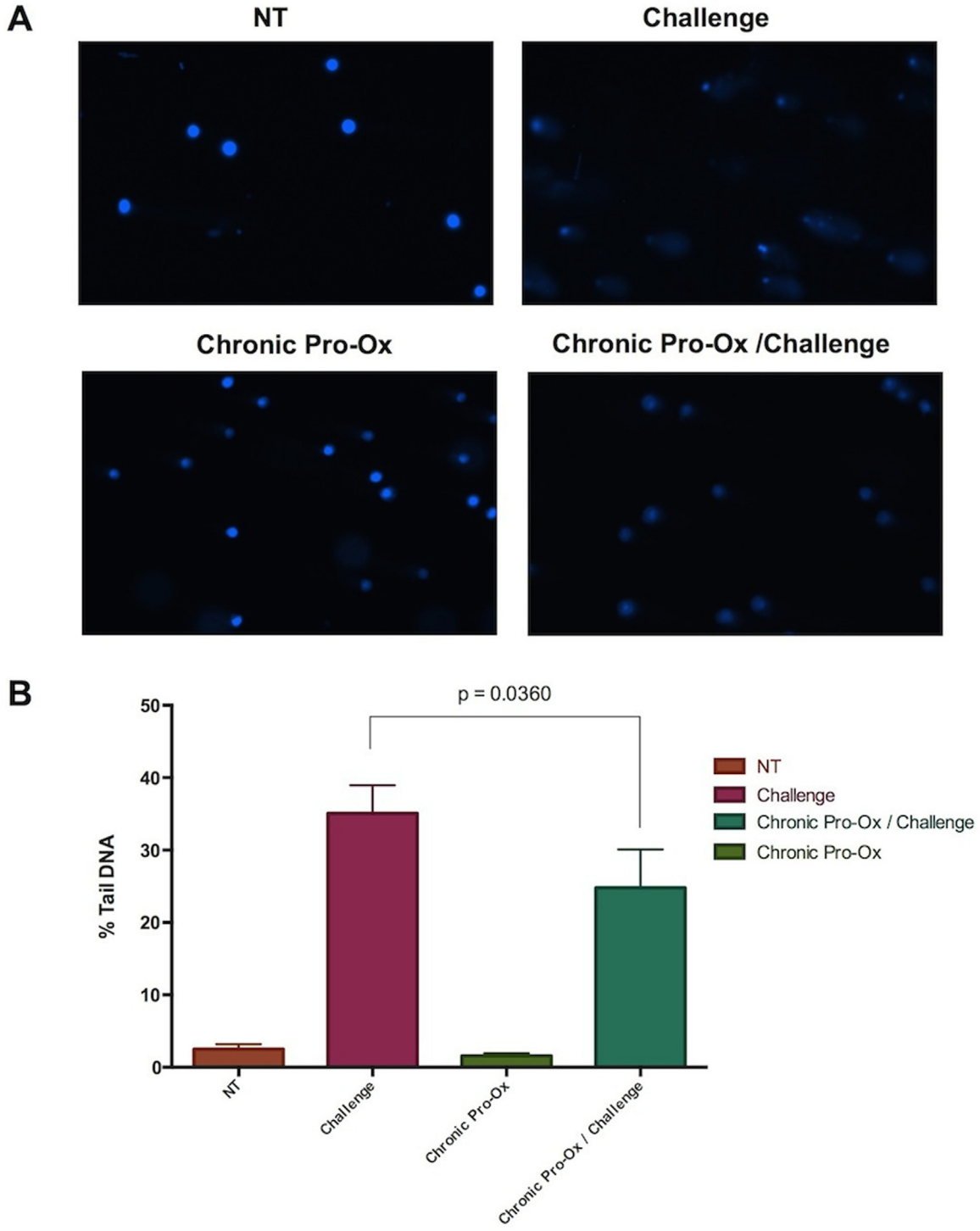

Fig. 2. Effect of chronic H_2O_2 pretreatments on DNA integrity in C2C12 cells. Cells were grown for seven days and pretreatments with chronic H_2O_2 dose were performed by a daily 1 h exposure to ~50 μM H_2O_2. On the last day of this process, cultures that had or had not undergone chronic H_2O_2 treatments were challenged with an acute dose of 0.5 mM H_2O_2 for 30 min, and subsequently processed by different tests to assess DNA integrity. Measurements of DNA strand breaks (mainly SSBs) were performed by DNA comet assay. (A) Representative images and (B) Quantification of tail DNA. Values are expressed as mean ± SEM of four independent experiments. Analysis of DSB was carried out by assessing chromosomal abnormalities after chronic and acute H_2O_2 treatments in C2C12 cells. (C) Pictures show representative examples of chromosomal abnormalities in each treatment, and (D) Quantification of clastogenic damage represented as % of damaged cells. Values are expressed as mean ± SEM. Note: C2C12 is a hyperdiploid cell line with a modal chromosome number of 71.

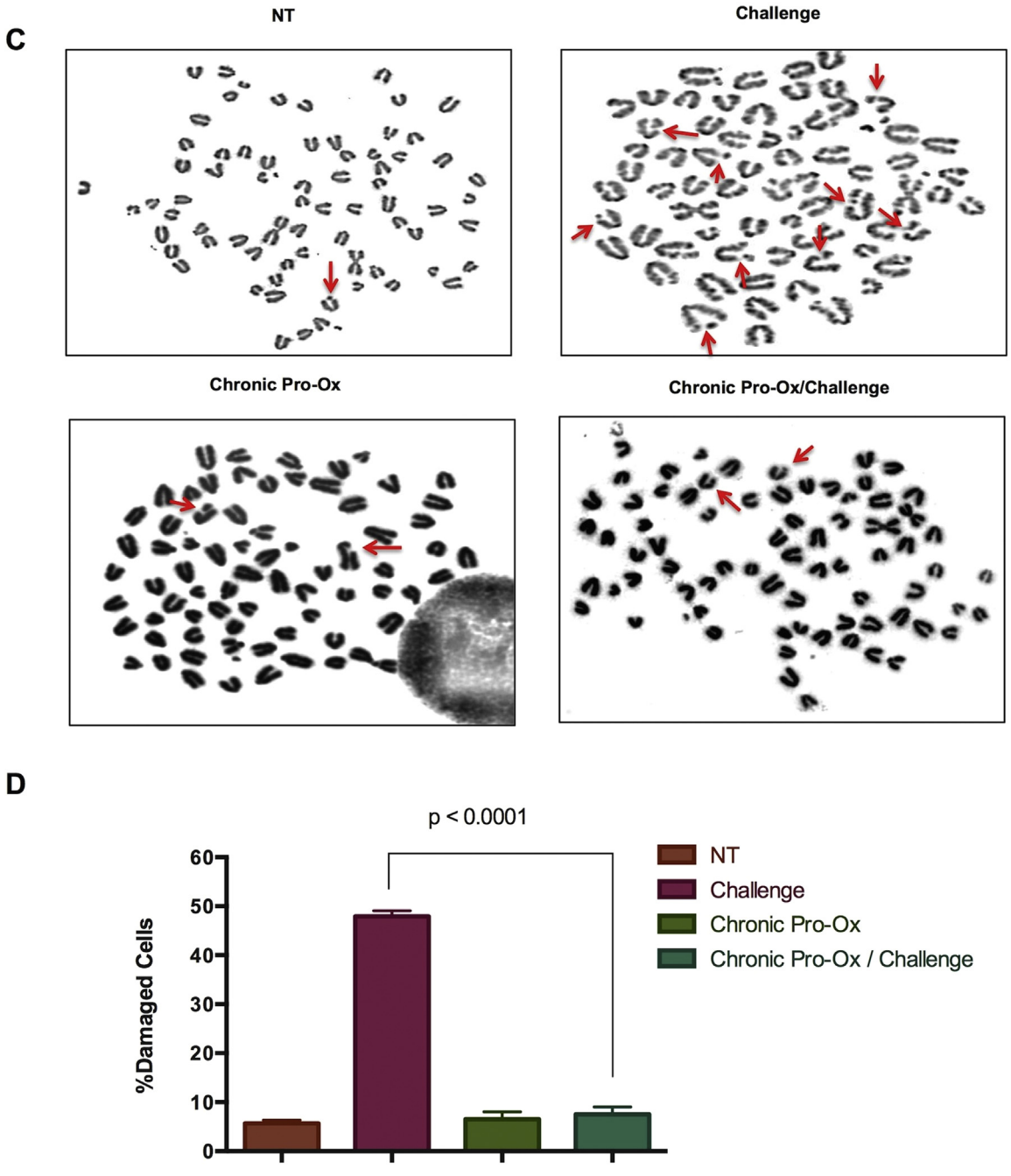

Fig. 2. (*continued*)

12 times higher after the challenge dose (Fig. 3B).

3.5. Long-term exposure to oxidative stimuli induces G2/M arrest

Since the difference in DNA damage did not seem attributable to increased kinetics of DNA repair, and since chromosomal damage is often accompanied by cell cycle disturbances, usually at specific check-points, we next tested whether chronic and challenge exposures affected cell cycle progression in C2C12 cells. Flow cytometric analyses of the C2C12 cell distribution in the absence of oxidant treatment identified a typical cell cycle distribution with most cells in G1 (57.3%). Compared to nontreated cells, chronically pre-treated cells showed an increased percentage of G2/M (from 20.9 to 41.1%; $p=0.0013$), as well as a decreased G1 phase (from 57.3 to 43%; $p=0.0123$) (Fig. 4). The challenge treatment (1 h), as expected, did not cause a statistically significant increase in G2/M phase (from 20.9 to 24.9%; $p=0.4319$). Most importantly, compared to challenged but not pre-treated cells, chronically pre-treated and challenged cells showed an increase in G2/M ($p=0.0176$). Thus, chronic pre-treatment resulted in cell cycle arrest that was not further altered by challenge treatment.

4. Discussion

Previous ROS adaptation studies have shown that a common response to cellular effects of oxidative stress after short experimental exposures and/or acute stress is the activation of antioxidant defense mechanisms. Fewer studies have utilized chronic, low-dose exposures, or examined genome homeostasis-related

Table 1
Differentially expressed genes in C2C12 cells after chronic pro-oxidant pretreatment, assessed by RT-PCR.

Gene symbol	Refseq	Fold changes	p	Significance	Definition
Apex1	NM_009687	2.3	0.0256	*	Apurinic/apyrimidinic endonuclease 1
Atr	NM_019864	3.0	0.0004	*	Ataxia telangiectasia and rad3 related
Brca1	NM_009764	2.5	0.9091	ns	Breast cancer 1
Brca2	NM_009765	2.7	0.5462	ns	Breast cancer 2
Ccnh	NM_023243	1.7	0.2581	ns	Cyclin H
Ercc1	NM_007948	2.1	0.0042	*	Excision repair cross-complementing rodent repair deficiency. complementation group 1
Ercc3	NM_133658	1.5	0.1779	ns	Excision repair cross-complementing rodent repair deficiency. complementation group 3
Ercc8	NM_028042	1.5	0.4018	ns	Excision repaiross-complementing rodent repair deficiency. complementation group 8
Exo1	NM_012012	1.9	0.1930	ns	Exonuclease 1
Fen1	NM_007999	1.5	0.3923	ns	Flap structure specific endonuclease 1
Lig1	NM_010715	2.7	0.8500	ns	Ligase I. DNA. ATP-dependent
Mlh1	NM_026810	1.9	0.0157	*	MutL homolog 1 (E. coli)
Mre11a	NM_018736	1.5	0.4226	ns	Meiotic recombination 11 homolog A (S. cerevisiae)
Msh2	NM_008628	1.6	0.0764	ns	MutS homolog 2 (E. coli)
Msh6	NM_010830	1.8	0.0379	*	MutS homolog 6 (E. coli)
Ogg1	NM_010957	1.8	0.0401	*	8-oxoguanine DNA-glycosylase 1
Parp3	NM_145619	1.7	0.0467	*	Poly (ADP-ribose) polymerase family. member 3
Pold3	NM_133692	1.6	0.3033	ns	Polymerase (DNA-directed). delta 3. accessory subunit
Rad18	NM_021385	2.3	0.0330	*	RAD18 homolog (S. cerevisiae)
Rad21	NM_009009	1.7	0.2362	ns	RAD21 homolog (S. pombe)
Rad50	NM_009012	1.7	0.2394	ns	RAD50 homolog (S. cerevisiae)
Rad51	NM_011234	1.8	0.2128	ns	RAD51 homolog (S. cerevisiae)
Rad51b	NM_009014	2.6	0.7908	ns	RAD51-like 1 (S. cerevisiae)
Rad54l	NM_009015	3.1	0.8920	ns	RAD54 like (S. cerevisiae)
Rfc1	NM_011258	2.1	0.0681	ns	Replication factor C (activator 1) 1
Rpa1	NM_026653	1.6	0.3299	ns	Replication protein A1
Top3a	NM_009410	1.5	0.4343	ns	Topoisomerase (DNA) III alpha
Xrcc1	NM_009532	1.8	0.0373	*	X-ray repair complementing defective repair in Chinese hamster cells 1
Xrcc6bp1	NM_026858	1.8	0.1959	ns	XRCC6 binding protein 1

ANOVA.
ns: not significant.
* Significantly different from control ($p < 0.05$).

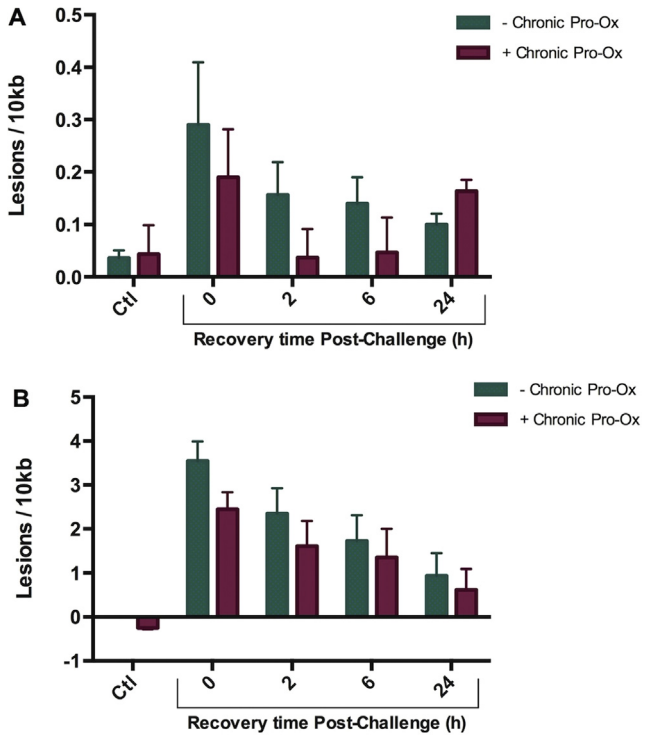

Fig. 3. Chronic, repeated H_2O_2 pretreatment reduces initial DNA damage after subsequent challenge, but does not detectably increase repair kinetics. The frequency of lesions was estimated by XL-QPCR immediately after the following time-points: 0 h, 2 h, 6 h and 24 h after challenge in both nuclear DNA (A) and mitochondrial DNA (B). For assessment of nDNA and mtDNA lesions, H_2O_2 concentrations of 1 mM and 0.5 mM were used, respectively.

processes. Using a pretreatment/challenge model in which we tested the ability of C2C12 cells to respond to a chronic and repetitive mild H_2O_2 stimulus, we found that these cells were able to tolerate very significant oxidative stress, evidenced by greatly reduced cytotoxicity, genotoxicity, and clastogenic damage. This tolerance was associated with an adaptive/cellular defense response that includes induction of DNA repair genes and activation of cell cycle arrest.

To assess the cytotoxic effects of the different treatments, we evaluated variations occurring in mitochondrial potential, which is one of the main markers of apoptosis-associated mitochondrial modification leading to cell death. As shown in Fig. 1B, increased mitochondrial trans-membrane potential, namely hyperpolarization of the inner mitochondrial membrane, was detected after a 1 mM H_2O_2 challenge concentration. This hyperpolarization phenomenon has been hypothesized to represent a very early change occurring in mitochondria during apoptosis [40–42]. Remarkably, the H_2O_2 pre-treatment exerted a significant protection from mitochondrial hyperpolarization; this might confer increased resistance to apoptosis.

Increased oxidative DNA damage from challenge with 1 mM H_2O_2 exposure resulted, as expected, in elevated ROS levels (Fig. 1D). In general, intracellular ROS may form oxidative base damage, apurinic (AP)-sites, SSB, and the most detrimental DNA injury, DSB. In our experimental model the challenge dose (1 mM H_2O_2) caused a marked induction of chromosome abnormalities. However, the chronic pre-treatment significantly reduced chromosome abnormalities, demonstrating a clear and specific protective effect against subsequent high-concentration H_2O_2 (Fig. 2B).

We identified increases in mRNA levels for genes involved in several pathways. For example, Atr is one of the major regulators of cell cycle arrest following DNA damage, and is essential for the

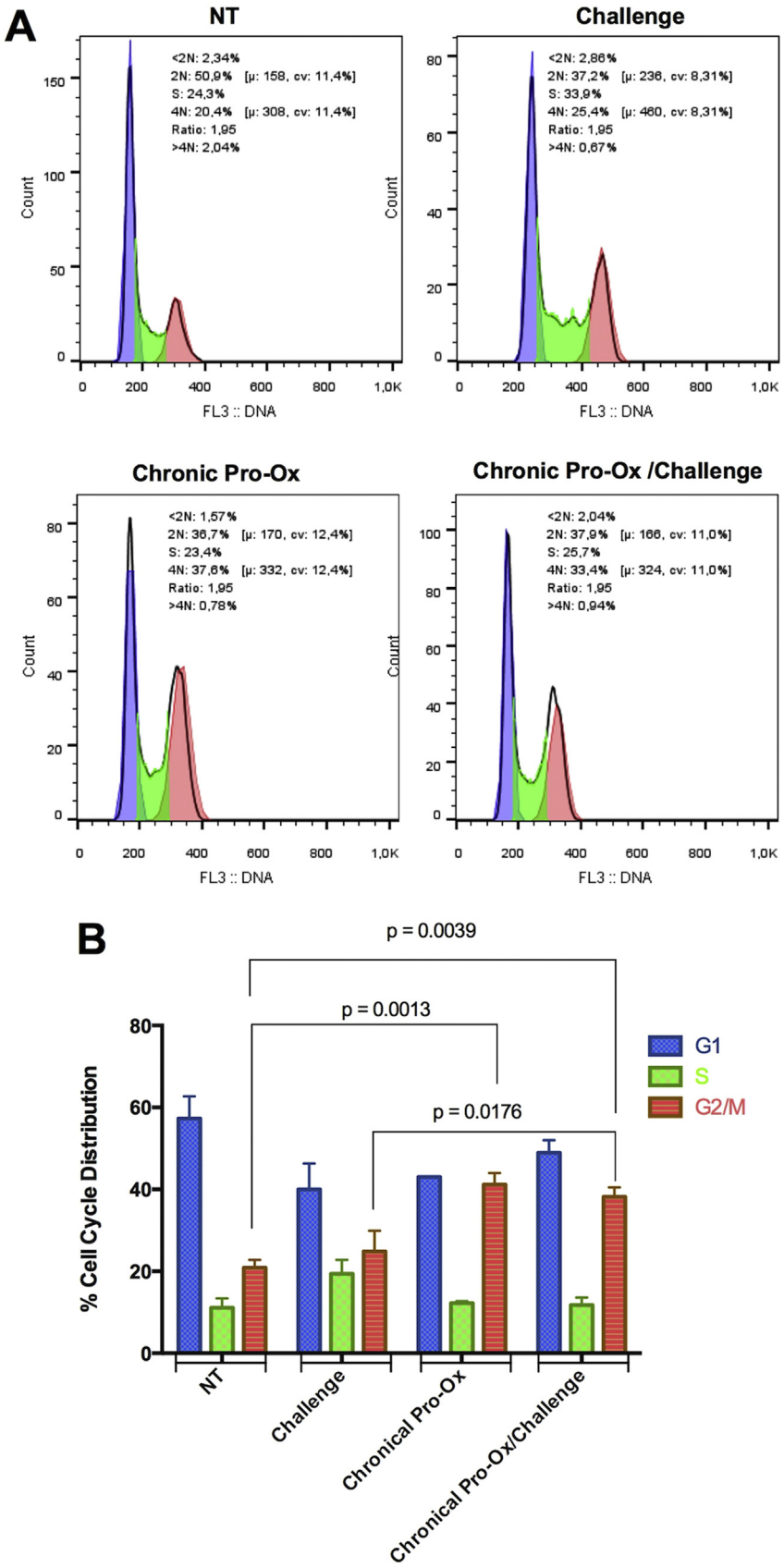

Fig. 4. Arrest of cell cycle progression at G2/M in response to chronic pro-oxidant treatment. (A) The histograms show representative examples of cell cycle phases distribution in each treatment. (B) Quantification of cell cycle distribution (%). Values are expressed as mean ± SEM of three independent experiments. * $p < 0.005$.

viability of replicating human and mouse cells [43]. Induction of Atr is thus in accordance with G2/M accumulation in C2C12 adapted cells. Rad18 is a protein required for activation of the ATR-dependent checkpoint pathway [44]. Ape1, Ogg1, Parp3 and Xrcc1 are involved in base excision repair (BER), which plays a key role by removing oxidative DNA damage, repairing single strand breaks, and repairing abasic sites [45,46]. Ercc1 is required to complete nucleotide repair (NER) and may also participate in the process of removing 8-OHdG lesions. Mlh1 and Msh6 are two key proteins in the mismatch repair (MMR) pathway, and have been implicated in the response to oxidative DNA damage, particularly 8-OHdG residues [47].

Despite increases in mRNA levels of several DNA repair genes, we did not detect increased rates of DNA repair in either genome, although initial levels of damage after challenge were lower in pre-treated cells. In fact, although the chronic pre-treatment protects against nDNA lesions at 0, 2 and 6 h of recovery time post-challenge, at 24 h we observed an increase of these lesions (Fig. 3). This could be due in part to unrepaired lesions that become postmitotically exacerbated due to replicative stress, like stalling, in the subsequent cell-cycle S phase. These assumptions and/or speculations are based on the following data: (a) It is well known that cells arrested at the G2/M repair checkpoint, usually prolong both phases depending upon the magnitude of damage, such that the originally arrested G2/M cell population could have reached S phase 24 h later. (b) Intrinsic leakiness of the G2 checkpoint has been documented [48], generating mitoses with unresolved DNA damages. (c) Recent reports have documented various mechanisms by which prolonged mitoses can result in additional kinds and amounts of DNA damage [49]. (d) Daughter cells from protracted mitoses progress differently through the following cell cycle [50]. Another way that cells might preserve genomic integrity is altered cell cycle progression, and in agreement with mRNA changes suggestive of cell cycle changes, we identified a G2/M phase accumulation with no major increase in cell death (Fig. 4). This cell phase-linked checkpoint is a filter-like response that impedes DNA-damaged cells from initiating mitosis. Therefore, our results suggest that the adaptation process includes induction of cellular response that permits repair and activation of genomic defense systems. Thus, although DNA repair kinetics *per se* may not be significantly altered, repair may be facilitated by cell cycle arrest.

This cell culture system may provide an *in vitro* model for the mechanistic study of any number of scenarios in which chronic, moderate oxidative stress is the norm. These could include chronic exposure to pollutants, or routine moderate exercise during which muscles acquire the capacity to withstand subsequent higher doses of ROS, such as those that occur during strenuous exercise. In an adaptation model like this, the main physiologic benefit could be to protect cells and possibly tissues against acute dosages of oxidant agents. Evidence shows that during such processes, cells increase enzymatic activities and pathways required to alleviate ROS [51]. In fact, current evidence indicates that exercise generates considerable amounts of ROS [52–54] that, in turn, may serve as a necessary "signal" for the up-regulation of endogenous antioxidant defense and repair systems [55–57], thereby providing protection against subsequent exposure to pro-oxidant environments [56].

Low levels of ROS may induce an adaptation process that resembles hormesis [58]. The hormesis theory proposes that biological systems respond to exposure to anthropogenic chemicals, toxins, and radiation in a bell curve-shaped fashion; it is part of a dose-response phenomenon characterized by low-dose stimulation and high-dose inhibition or saturation of homeostatic processes [59]. The stimulating "toxin" during exercise is ROS formation, which increases resistance to oxidative stress [58].

In summary, we found that long-term intermittent exposure to oxidative stimuli induces adaptive behavior, which permits mammalian cells to decrease ROS accumulation and oxidative DNA damage in association with cell cycle arrest. This DNA damage response is likely due at least in part to up-regulation of a broad spectrum of DNA repair and cell cycle arrest proteins that mediate effective activation of the defense system against acute challenges.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.redox.2016.07.004.

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