Vol. 210, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 5, 1995 Pages 189-196

LOW-LEVEL OXIDATIVE STRESS CAUSES CELL-CYCLE SPECIFIC ARREST IN CULTURED CELLS

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Received March 29, 1995

Oxidative stress is involved in a multitude of pathological conditions. In the present study, cell cycle arrest was demonstrated in monolayer cultures of Chinese hamster ovary cells subjected to low-level oxidative stress induced by the addition of hydrogen peroxide. Fluorescence-activated cell sorting analysis characterized this arrest as occurring in both the G_1 and G_2/M phases of the cell cycle. Upon exposure to oxidative stress, cells in G_1 arrest in G_1, while S-phase cells complete DNA synthesis and subsequently arrest in G_2/M. Nitroxide spin labels with differing permeabilities are able to protect the cells, which suggests that both the G_1 and the G_2/M arrest involve oxidation of targets located at the cell surface.

The exposure of both quiescent and actively dividing cultured cells to low levels of hydrogen peroxide (H_2O_2) has been shown to lead to growth arrest (1, 2). This arrest occurs in the absence of such classic signs of cellular damage as DNA oxidation, loss of metabolic capacity, membrane disruption, apoptosis, or necrosis. Confluent human diploid fibroblasts exposed to low levels of H_2O_2 demonstrate an inability to re-enter the cell cycle and initiate DNA synthesis in response to mitogenic stimuli (2).

Exposure of actively dividing cells to X-ray or ultraviolet (UV) radiation arrests cells in both G_1 and G_2 of the cell cycle (3). Cells exposed to radiation during S-phase delay completion of DNA synthesis, but ultimately arrest in G_2, whereas cells irradiated during G_1 arrest in G_1. Cell cycle checkpoints are involved in the cellular arrest. The *p53* gene product seems to play a major role in the G_1 arrest (3-5). The mechanism underlying the G_2 arrest, however, has not been elucidated, although regulation of cyclin B1 and/or $p34^{cdc2}$ has been suggested (6). Presumably

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0006-291X/95 $5.00

the arrests allow the cells to repair damage and prevent permanent DNA modification. Alternatively, apoptosis may occur as a result of irradiation or chemical oxidative stress.

Cells vary in their sensitivity to physical and chemical agents over the course of the cell cycle. Chinese hamster ovary (CHO) cells exposed to cisplatin while in G_1 evidence considerably greater cell death and chromosomal aberrations than do cells exposed during S-phase (7). Survival of X-irradiated cells is dependent on the position of the cell in the cell cycle (8). The increased susceptibility of tumor cells to radiation in the presence of some radiosensitizers is partially due to an enrichment of early S-phase cells (9). The duration of the G_2 arrest of synchronized CHO cells increases as cells are irradiated later in the cell cycle (10).

The present study extends our previous work with oxidative stress in actively dividing CHO cell populations (1). Fluorescence–activated cell sorting (FACS) analysis reveals that low–level oxidative stress leads to an arrest in both G_1 and G_2/M of the cell cycle. CHO cells exposed to H_2O_2 while in G_1 remain in G_1, while S-phase cells complete DNA synthesis and arrest in G_2/M. Both the G_1 and G_2/M arrests appear to be mediated by events occurring at the cell surface.

Materials and Methods

Materials: N,N,N-trimethyl-4-ammonium-2,2,6,6-tetramethylpiperidine-N-oxyl, iodide (CAT1), 12–doxylstearic acid and 4-(N,N-dimethyl-N-hexadecyl) ammonium-2,2,6,6-tetramethylpiperidine-1-oxyl, iodide (CAT 16) were obtained from Molecular Probes, and 2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPO) from Aldrich. Tween–20, Triton X–100, propidium iodide, bromodeoxyuridine (BrdUrd), were from Sigma. Anti-BrdUrd was from Boehringer-Mannheim. Fetal bovine serum was from Gibco BRL. Fungizone, trypsin, phosphate buffered saline (PBS) and Dulbecco's Modified Eagle's Media (DME) were from the UCSD CORE Cell Culture Facility. Gentamycin was from Gemini Bioproducts. Non–essential amino acid solution was from Irvine Scientific.

Cell Culture and Synchronization: CHO cells were grown in DME, regular glucose, supplemented with 10% fetal bovine serum, 1% non–essential amino acids, 1% Fungizone and 0.4% gentamycin. Cultures were grown to mid log phase in 10 cm dishes, and used at densities ranging from 1.5 to 4 x 10^6 cells per plate. Serum starvation was used to synchronize the cells. After cells were plated and allowed to attach overnight, the medium was replaced with DME containing 0.1% fetal bovine serum. Forty–four hours later, the medium was replaced with fresh DME containing 10% fetal bovine serum and 1% non–essential amino acids. The cells were exposed to oxidative stress approximately 5.5 hours later (G_1–phase exposure) or 12 hours later (S-phase exposure).

Oxidative Stress: Previous studies have shown that damage due to oxidative stress depends on the amount of oxidant per cell (1, 11, 12). Oxidative stress was therefore induced by incubating cells with 0.4 μmoles $H_2O_2/10^6$ cells in DME high glucose without pyruvate for 1 hour at 37°C. This level of H_2O_2 results in a 90 to 95% decrease in the reproductive survival of CHO cells with no observed metabolic, DNA or membrane damage (1). Where stated, the nitroxides TEMPO and CAT1 were added at 1 and 20 mM respectively. The final experimental volume was 5 mL. After treatment, the medium was removed, the cells rinsed, and then incubated in supplemented medium for up to 48 hours.

Flow Cytometry and Sorting: After treatment and incubation, the cells were exposed to medium containing 20 μM BrdUrd for 30 minutes. Cells were harvested and prepared for flow cytometry essentially by the method of Dolbeare, *et al.* (13). Briefly, cells were stored overnight at 4°C in 70% ethanol, removed from the ethanol and resuspended in 2.5 M HCl with 0.5% Triton X–100 for 25 minutes at room temperature. Cells were then washed twice in 0.5% Tween–20 in PBS, resuspended for 30 minutes in PBS containing 0.5% Tween–20, 1.5% (w/v) non–fat dry milk, and anti–BrdUrd, washed twice with PBS, and finally resuspended in 0.5 mL of PBS containing 4 μg/mL propidium iodide, 0.1% Triton X–100, and 0.2 mM EDTA. Cell sorting was performed using a FACScan Flow Cytometer (Becton–Dickinson Immunocytometry Systems). Data analysis was performed using the WinMDI version 1.3.1 flow cytometry analysis utility (Joe Trotter, Salk Institute) and Multicycle version 2.50 (Phoenix Flow Systems).

Results and Discussion

A persistent accumulation of cells in any stage of the cell cycle was taken as evidence for an arrest in that stage of the cycle. Exposure of CHO cells to low–level oxidative stress leads to an arrest in both G_1 and G_2/M of the cell cycle (Table 1). Not all cells exposed to this level of H_2O_2 arrest. After 48 hours, the 7% of the cells which remain reproductively viable have replicated several times, resulting in the presence of S-phase cells. Cells exposed to H_2O_2 arrest at both cell cycle checkpoints suggesting that specific mechanisms are involved in the growth arrest.

Previous work with nitroxides implicated surface–mediated mechanisms in the growth arrest (1). Two nitroxides were found to provide partial protection: CAT1, which is restricted to the extracellular spaces even under the conditions used in these experiments, and TEMPO, which freely enters cells (1, 14). We were interested in determining whether the partial

Table 1. The effect of low–level H_2O_2 on the cell cycle distribution of CHO cells

Treatment	Percent of Cells in		
	G_1	S	G_2 / M
Control	27.5	59.4	13.1
H_2O_2	40.8	12.1	47.1
H_2O_2 + 1 mM TEMPO	34.4	46.0	19.7
H_2O_2 + 20 mM CAT1	33.8	54.0	12.2

Asynchronous populations of CHO cells were incubated 60 min at 37°C with 0.4 μmoles $H_2O_2/10^6$ cells and 1 mM TEMPO or 20 mM CAT1, as indicated. The medium was then replaced with supplemented medium. Forty-eight hours later, cells were prepared for flow cytometry and their distribution in the cell cycle was determined. Results are the mean of three independent experiments.

protection was due to the prevention of arrest at only one of the checkpoints. For example, protection of only S-phase cells could lead to the 40% survival seen previously (1). Our results demonstrate that both CAT1 and TEMPO prevent arrest at both checkpoints (Table 1). Exposure of the cells to either of the nitroxides in the absence of H_2O_2 did not lead to any differences in the cell cycle distribution when compared to controls (data not shown). The protective efficacy of the impermeable CAT1 suggests that both arrest mechanisms are initiated at the cell surface.

The ability of two other nitroxides to protect against exposure to H_2O_2 was evaluated. 12–doxylstearic acid, which partitions into the membrane in a configuration which inhibits lipid peroxidation (15), provided no protection. Limited protection was provided by CAT 16, which is an amphipathic nitroxide with a hydrophilic catalytic head that remains outside the membrane. At non–toxic levels, use of CAT 16 resulted in a clonogenic survival rate of 20% compared to the 40% of CAT1 and TEMPO. The failure of 12–doxylstearate to provide protection suggests that membrane lipids are probably not the primary target of low–level oxidative stress. The results with CAT1 and CAT 16 argue that the initial site of cellular damage is located at the outer surface of the plasma membrane.

Cellular response to UV exposure involves the membrane. The transcription factors NF–κB and AP–1 are activated as part of the UV response. NF–κB is stored as an inactive cytoplasmic complex, and is dissociated and activated in response to a variety of stimuli, including UV exposure. Exposure of enucleated cells to UV leads to NF–κB activation (16). UV irradiation of crude cytosolic extracts shows that activation requires the presence of plasma membranes (17). The activation of the transcription factor AP-1 after UV exposure partially depends upon functional growth factor receptors (18). While the actual mechanisms may differ, there appears to be a central role of the plasma membrane in the response both to UV and to H_2O_2.

Synchronized cells were used to determine if phases of the cell cycle were differentially sensitive to oxidative stress. There was no difference in the reproductive survival of cells treated in G_1 and those treated in S-phase. CHO cells exposed to H_2O_2 during G_1 largely remain in G_1 (Table 2). Although no DNA synthesis was indicated in cells harvested immediately after H_2O_2 exposure, some cells arrested in G_2/M. These cells probably passed the $G_1 \rightarrow$ S restriction point, which occurs well before the end of G_1, prior to H_2O_2 exposure. Cells treated during S-phase arrest in G_2/M (Table 2). Although the data presented are from cells collected 24 hours after treatment, the results changed little after 48 hours other than to reflect the increasing population of unarrested cells. These results suggest a failure to progress past specific cell cycle checkpoints once damage has occurred (19).

Table 2. The effect of H_2O_2 exposure during G_1 or S on the subsequent cell cycle distribution of CHO cells

Treatment	Percent of Cells in G_1	S	G_2/M
G_1 Exposure			
Control	35.8	52.0	11.3
H_2O_2	67.4	9.3	21.8
S Exposure			
Control	9.3	81.5	9.2
H_2O_2	1.7	6.2	92.1

Synchronous populations of CHO cells were incubated 60 min at 37°C with 0.4 μmoles $H_2O_2/10^6$ cells. The medium was then replaced with supplemented medium. Twenty-four hours later, cells were prepared for flow cytometry and their distribution in the cell cycle determined. Results are the mean of three independent experiments.

Chen and Ames reported that confluent human diploid fibroblasts exposed to low-level oxidative stress were unable to synthesize DNA (2). These cells were presumably in G_0 at the time of exposure to H_2O_2. Our results show that DNA synthesis is completed if the cell has passed the $G_1 \rightarrow S$ checkpoint prior to oxidative stress. Cells stressed during S-phase do complete DNA synthesis, although they lag behind control cells (Figure 1). This is consistent with the results of Fiorani, *et al.*, who documented decreased rates of DNA synthesis in CHO cells exposed to low levels of H_2O_2 (12).

The FACS data confirm that apoptosis is not occurring in these cells. None of the H_2O_2-treated samples displayed the distinct sub-G_0/G_1 peak characteristic of apoptotic cells (20, 21). Samples harvested up to 4 days after exposure to H_2O_2, by which time unarrested cells probably constituted greater than 80% of the total cell population, still showed no sub-G_0/G_1 peak.

The procedure of Gelvan, *et al.* (1) was used to determine if other cell lines would also demonstrate a growth arrest at low levels of oxidative stress. Four cell types were tested: two NIH3T3 subclones (1A4- and 4A2+), SAOS, and human foreskin fibroblasts. All four yielded results similar to those obtained with CHO cells, differing only in the levels of H_2O_2 per cell required to cause arrest or metabolic failure (data not shown). Both 3T3 cell lines require a high level of H_2O_2 to manifest arrest—these concentrations approach levels which cause metabolic failure in these cells. This is most likely due to the mitogenic influence of H_2O_2 on 3T3 and other murine cell lines (22-25).

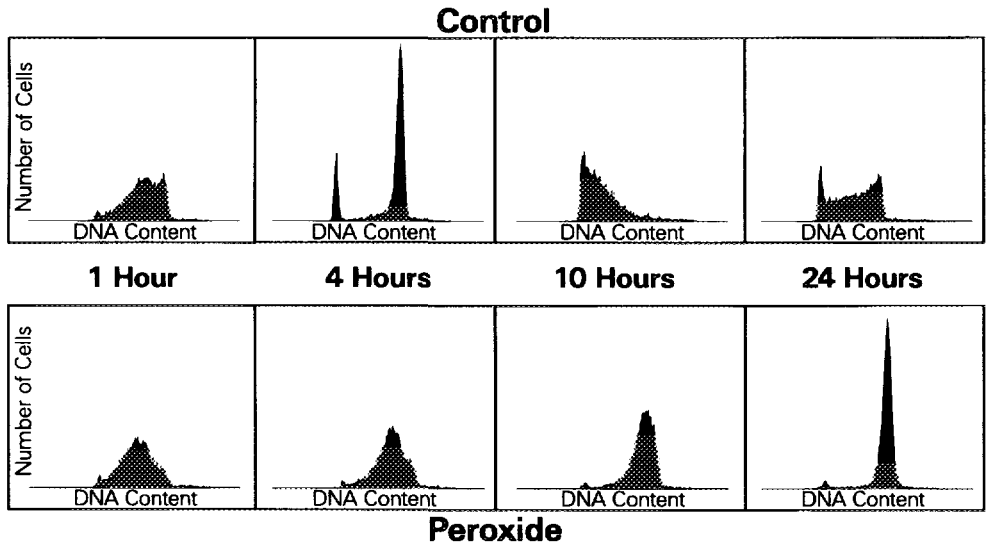

Figure 1. Cell cycle distribution of S Phase CHO cells exposed to H_2O_2. CHO cells synchronized in S phase were incubated 60 min at 37°C with 0.4 μmoles $H_2O_2/10^6$ cells. The medium was then replaced with supplemented medium. At the indicated times, cells were prepared and analyzed by flow cytometry.

The mechanisms underlying growth arrests following exposure to radiation are poorly understood. Previous experiments involving radiation show that the G_1 arrest is probably due to p53 (4, 5). SAOS cells, which do not express p53, arrest in G_1 in response to H_2O_2 exposure. Cells exposed to radiation exhibit a stable increase in p53 DNA binding in contrast to the transient binding seen in peroxide treated cells (26). Thus, p53 is probably not the primary agent in G_1 arrest caused by oxidative stress. The G_2 arrest in response to ionizing radiation may involve regulation of cyclin B1 and/or $p34^{cdc2}$ (27, 28). However, at the present time we have no evidence to implicate either of these factors in the G_2 arrest due to H_2O_2.

CL100 and its murine homolog MAP Kinase Phosphatase 1 (MKP-1) may be part of the cellular response to oxidative stress. There is a pronounced induction of CL100 mRNA in human skin fibroblasts subjected to relatively low concentrations of H_2O_2. Levels of CL100 mRNA were 15 times higher in H_2O_2 treated cells than in X-radiated cells (29). Injection of an oncogenic mutant of Ras (V^{12} Ras) into quiescent rat embryonic fibroblasts leads to the initiation of DNA synthesis. This is blocked by coinjection of an expression plasmid for MKP-1 (30). Both proteins specifically dephosphorylate MAP Kinase (31, 32). This activity is probably responsible for blocking the effects of V^{12} Ras and might also help explain the G_1 arrest reported here.

There is a similarity between the growth arrest of cells exposed to UV or X-radiation and cells exposed to low-level oxidative stress induced by H_2O_2 and superoxide (1). In oxidatively

stressed cells, there appears to be a specific inhibition which prevents passage through cell cycle checkpoints. These results are manifest across different cell lines. Although DNA synthesis is slowed, cells that have passed the $G_1 \rightarrow S$ checkpoint complete DNA synthesis. Growth arrest thus appears to be a function of a specific cellular injury, not a general failure of the cellular machinery. The phenomena of cell arrest observed with both UV radiation and with oxidative stress requires the participation of plasma membrane components and signalling mechanisms which prevent cells from proceeding through the cell cycle.

Acknowledgments

The authors thank Dr. Martin Haas for his thoughtful advice early in this project and for providing SAOS and human fibroblast cells, and Dr. Jean Wang for providing 3T3 cells. We also thank Jo Yeargin and Joe Trotter for their useful advice and assistance in performing FACS, and Elizabeth Machunis for her advice in preparing this manuscript. This work was supported by a gift from the Proctor and Gamble Co. D.A.C. was supported by a National Institute of Health Training Grant (NIH 5T32GM07313).

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