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Sites and mechanisms of low-level oxidative stress in cultured cells

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Oxidative stress is involved in a multitude of pathological conditions. In the present study, we investigated the cellular targets and the mechanisms of low-level oxidative stress in a Chinese Hamster Ovary cell culture. Oxidative stress was induced either by continuous enzymatic production of superoxide or by bolus addition of hydrogen peroxide (H_2O_2). Low-level oxidative stress irreversibly impaired the reproductive capacity of the cells in the absence of damage to membrane integrity or energy metabolism. Cells were protected by catalase but not by superoxide dismutase, indicating that H_2O_2, not superoxide, was the causative agent of cell damage. Nitroxide spin labels decreased hydroxyl radical (•OH) formation and protected cells from the oxidative stress. The differing membrane permeabilities of these spin labels suggest that the damage is localized on the cell surface. Oxidative stress to DNA and RNA was not significant, as shown by levels of guanine hydroxylation products. A mechanism is proposed whereby low-level oxidative stress acts at the cell surface to cause impairment of cell reproduction.

Oxidative stress has been implicated in a wide range of disease states and degenerative processes, such as cardiovascular disease, cancer, rheumatoid arthritis, inflammatory states, ischemia/reperfusion states and aging (1). The exact mechanisms of free radical insults to biological systems are not well defined. One strategy to prevent damage has been the enzymatic removal of reactive oxygen species, *i.e.* superoxide ($O_2^{\cdot-}$) by superoxide dismutase (SOD) or H_2O_2 by catalase (2, 3). The search for an SOD based therapy is predicated on the ability of $O_2^{\cdot-}$ to reduce metal ions and for the latter to react with H_2O_2 forming hydroxyl radical (•OH) in the Haber-Weiss reaction (4). The efficacy of SOD in reducing free radical damage has been inconsistent (2, 3 and references therein). This ambiguity may be a function of the lack of participation of $O_2^{\cdot-}$ in the reduction of metal ions, or the failure of SOD to access the sub-cellular target of the primary attack.

A group of nitroxide spin-labels protect against oxidative stress in both cellular systems (5, 6) and in isolated organs (7). In general, these antioxidant nitroxides all contain an >N-O• radical with blocked α-carbons, which determines the catalytic activity. The remainder of the molecule determines other properties,

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including biological distribution. Thus, nitroxides with low polarity, such as 2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPO), rapidly enter cells, whereas charged nitroxides such as N,N,N-trimethyl-4-ammonium-2,2,6,6-tetramethylpiperidine-N-oxyl, iodide (CAT1) do not cross cell membranes (8).

The present study shows that low level oxidative stress selectively interferes with cell proliferation. Previous investigators have documented that at relatively high levels of oxidative stress, membrane disruption, loss of vital staining, and DNA oxidation are among the pathologies induced. We find that low-level oxidative stress decreases the reproductive capacity of Chinese Hamster Ovary (CHO) cells, without affecting their membrane integrity or metabolic function. Although not identified, the primary target of this low-level oxidative stress appears to be located on the cell surface. The damage is H_2O_2 dependent and insensitive to removal of $O_2^{\cdot-}$.

Materials and Methods

Materials. CAT1 was from Molecular Probes. TEMPO, trypan blue and potassium-tris(oxalato)chromate (CROX) were from Aldrich. SOD (EC 1.15.1.1), bovine catalase (EC 1.11.1.6, SOD free), hypoxanthine (HX), xanthine oxidase (XO), and Giemsa stain were from Sigma and 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, and non-essential amino acid solution was from Irvine Scientific. 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 12 well plates and used at a density of approximately $5 \cdot 10^5$ cells per well.

Experimental conditions. Oxidative stress was induced by incubating cells in DME high glucose without pyruvate for 1 hour at 37°C with 0.1 mM salicylate and either: 0.4 μmoles $H_2O_2/10^6$ cells or 1 mM HX with 0.006 units XO/10^6 cells (HX/XO). Several antioxidants were added individually: SOD, catalase, TEMPO or CAT1. The final volume was 0.5 ml/well. After incubation, the medium was removed for determination of lactate dehydrogenase (LDH) or oxidized DNA. The cells were harvested for the determination of colony forming ability, trypan blue exclusion and DNA oxidation. Similar experiments were performed in PBS with 0.45% (w/v) glucose and 0.1 mM salicylate since DME prevented salicylate-based detection of •OH formation (D. Clopton, D. Gelvan and P. Saltman, unpublished results). These experiments were limited to 30 min. to minimize cellular metabolism of salicylate to 2,5-dihydroxybenzoic acid (9). The supernatants were collected for determination of dihydroxybenzoic acid (DHBA).

Colony forming ability. After treatment, cells were plated at 100 or 1000 cells/well in 6 well plates, incubated for 7 days, fixed with ethanol:acetic acid (3:1), stained with Giemsa stain, and the colonies counted.

Trypan blue exclusion. At various times up to 7 days after treatment, cells were stained with trypan blue, 1:10, and counted using a hemocytometer.

LDH release. LDH in the supernatants was determined essentially as described by Bergmeyer *et al.* (10). Total cellular LDH was determined by assaying lysed control cells. LDH release was calculated as percent of total cellular LDH.

Determination of DHBA. Samples were analyzed using HPLC and electrochemical detection. DHBAs were separated on a Microsorb C18 column (5 μm spherical bead diameter, 4.6 mm i.d. x 15 cm length) using a mobile phase containing 1% acetic acid and 45% methanol (v/v) in water at pH 2.9 at a flow rate of 0.9 ml/min. DHBAs were detected by electrochemical detection using the following parameters: guard cell, +0.55 V; detector 1, +0.05 V; detector 2, -0.30 V. Concentrations were calculated from a standard curve.

Electron Spin Resonance. Cells were concentrated to 10^8/ml in high glucose DME and mixed with 1 mM TEMPO or 5-20 mM CAT1. Where stated, 0.5 M CROX and/or 40 mM H_2O_2 were added. Samples were introduced into 15 cm long gas-permeable teflon capillaries (Zeus Industries, Raritan, NJ) of 0.032 inch internal diameter, 0.015 inch wall thickness. Each capillary was folded twice, inserted into open ended quartz ESR tubes and placed in the cavity of a Bruker ESP300 Electron Spin Resonance spectrometer. Samples were scanned several times in the course of an hour, using the following settings: Microwave frequency 9.51 GHz; microwave power 2 mW; modulation frequency 100 kHz and modulation amplitude 0.26 G.

DNA and RNA oxidation. Cells were lysed, and nuclei collected and prepared. Levels of 8-hydroxy-2'-deoxyguanosine (oxo^{8}dG), 8-hydroxyguanine (oxo^{8}Gua) or 8-hydroxyguanosine (oxo^{8}G) in cells and medium were determined using HPLC and electrochemical detection, as described by Shigenaga *et al.* (11).
Rate constants for the reaction of TEMPO, CAT1 and SOD with $O_2^{\cdot-}$. The second order rate constants for the reaction of TEMPO, CAT1 and SOD with $O_2^{\cdot-}$ were determined essentially as described by Samuni *et al.* (5). Calculations for TEMPO were performed with data from the linear region only (12).

Results and Discussion

Mechanism of cell damage.

Two modes of oxidant administration were employed to characterize the effects of oxidative stress on cultured CHO cells: continuous extracellular production of $O_2^{\cdot-}$ by HX/XO or bolus administration of H_2O_2. Several parameters of cell function were measured: clonogenic activity (reproductive survival), trypan blue exclusion ("metabolic survival") and LDH release (membrane integrity). CHO cells exposed to a range of concentrations of H_2O_2 exhibited reproductive damage at low concentrations, while metabolic and membrane damage occurred only at 20 fold higher concentrations (Fig. 1).

Culture plates containing identical cell numbers were incubated with varying volumes of DME containing a fixed concentration of H_2O_2 (0.5 mM) or HX/XO (1 mM; 0.005 units/ml). Cell damage increased with increasing amounts per cell. The effect of the oxidative stress depends on the amount of oxidant per cell, not the concentration. This suggests that a cellular factor participates in the mechanism of cell damage by the oxidants or in its prevention. The dependence of H_2O_2's toxicity on cell density may result from its removal by cellular catalase (13). At higher cell densities H_2O_2 will be removed more rapidly, thereby reducing the effective exposure of the cells to H_2O_2. Alternatively, a threshold number of "hits" per cell may be required to block cellular reproduction. This is supported by the close correlation observed between the consumption of H_2O_2 and reproductive impairment (D. Clopton and P. Saltman, unpublished results). Subsequent

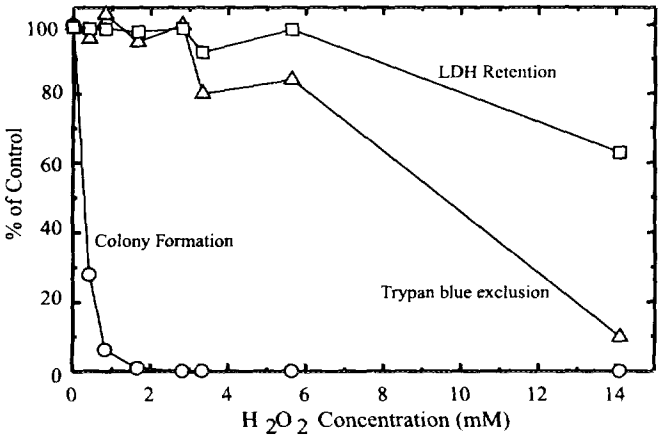

Figure 1. The effect of H_2O_2 concentration on cell damage. CHO cells were incubated 60 min at 37°C with varying concentrations of H_2O_2 and trypan blue exclusion (△), LDH retention (□) and colony forming ability (○) were determined. A typical experiment, representative of three similar repeats, is shown.

experiments used 0.4 μmoles H_2O_2 or 0.006 units XO per 10^6 cells. A 60 min. exposure at these levels caused a 90-95% decrease in the reproductive survival of the cells but no observable metabolic or membrane damage.

Cells were exposed to either H_2O_2 or $O_2^{\cdot-}$ (HX/XO) in the presence of SOD or catalase. SOD provided no protection (Table 1). This suggests that extracellular $O_2^{\cdot-}$ is not directly involved in the mechanism of cell damage, even when $O_2^{\cdot-}$ is the primary oxidant source. Catalase completely protected cells, indicating that the damage induced by both systems is directly dependent on H_2O_2. Both SOD and catalse are restricted to the extracellular space; it is unlikely that SOD's failure was due to lack of access to the oxidants.

The antioxidant nitroxides share common mechanisms of action (14), but distribute differently between cell compartments. TEMPO equilibrates freely into cells, whereas CAT1 is exclusively extracellular (8). The antioxidant activity of the nitroxides is potentially a function of four different reactions: a) disproportionation of $O_2^{\cdot-}$ (5, 6); b) oxidation of reduced transition metals required for cleavage of H_2O_2 (6); c) reaction with organic radicals produced as reactive intermediates in biological damage (15); and d) direct reaction with •OH. Nitroxides do not possess catalase-like activity (14).

The second order rate constants for the reaction with $O_2^{\cdot-}$ were: SOD - $1.2 \pm 0.4 \times 10^9$; TEMPO -$8.1 \pm 0.6 \times 10^5$; and CAT1 - $5.4 \pm 0.4 \times 10^4$. Nitroxides and SOD were used at roughly equiactive concentrations of the compounds, *i.e.* 1 mM TEMPO, 20 mM CAT1 and 46 units SOD/ml. TEMPO and CAT1 provided considerable protection to the cells, increasing survival from 5% to approx. 40% at the concentrations used, Table 1. Further protection could be obtained by increasing the nitroxide concentrations (data not shown). The effects of a combination of TEMPO and CAT1 were additive. It is unlikely that nitroxides protect through their SOD-mimicking activity, since they also protect against H_2O_2-induced damage, Table 1 and others (6, 14). It remains to be determined whether nitroxides protect the cells by oxidizing catalytic metal ions, scavenging •OH, or by detoxifying secondary organic radicals.

Ferric nitrate in DME could enhance oxidative damage to the cell surface, and explain CAT1's protective effect. However, it is highly unlikely that this iron is reactive (16). Alternatively, CAT1 could prevent the

Table 1. The effect of SOD, catalase, TEMPO and CAT1 on reproductive survival of CHO cells subjected to oxidative stress

	Reproductive survival (% of Control)[a]	
Antioxidant	H_2O_2-treated	HX/XO-treated
None	4.4±1.7	4.6±0.5
1 mM TEMPO	42.1±4.0	34.8±4.1
20 mM CAT1	41.1±5.5	34.0±4.6
None	10.9±1.3	9.3±6.3
SOD (46 U/ml)	10.3±0.7	7.9±4.9
None	3.6±1.1	6.6±1.7
Catalase (10 U/ml)	101.0±5.8	98.4±2.9

[a] Mean ± SD of three independent determinations.

formation of secondary radical species which then enter the cell. Glucose-supplemented PBS used in place of DME led to no change in results obtained with or without CAT1 (data not shown), suggesting that secondary radicals in the medium are probably not involved.

Exposure to oxidative stress could render cells permeable to CAT1. This was investigated by ESR. The strictly extracellular CROX broadens the ESR signals of TEMPO and CAT1 without interacting chemically with these radicals (17). When both CROX and TEMPO were added to cells, a residual signal was observed, corresponding to intracellular TEMPO. Addition of CROX to cells and CAT1 completely eliminated the CAT1 signal, confirming that CAT1 did not penetrate into the cells to any appreciable degree. Under oxidative stress produced by 0.4 μmoles H_2O_2 per 10^6 cells for 1 hour, the signal from intracellular TEMPO was identical to that from untreated cells. Thus, oxidative stress did not render the cells permeable to CROX. When the experiment was repeated with CAT1, no signal was observed, indicating that no measurable CAT1 penetrated into the cells. Nitroxides undergo intracellular metabolism (18). The amplitude of the signal from TEMPO decreased with time, whereas the signal from CAT1 remained stable. H_2O_2 had no effect on the cellular metabolism of the nitroxides. The absence of metabolism of CAT1 constitutes further evidence that this nitroxide did not enter the cells. CAT1 protected the cells while remaining extracellular.

The dependence of cell damage on H_2O_2 may implicate •OH as the ultimate oxidant in cell damage. The conversion of salicylate to DHBA is a measure of •OH formation. Treating cells with either H_2O_2 or HX/XO caused a large increase in DHBAs, Table 2. Samples containing CAT1 could not be analyzed as its redox peak obscured the DHBA peaks. TEMPO decreased DHBA levels to well below control values while affording only partial protection to the cells. The sites of •OH production may be relatively inaccessible to salicylate, which may be a poor reporter of biologically important site-specific radical formation (19). Alternatively, other oxidant species such as hypervalent metals, may be involved in the causation of cell damage.

The primary site of oxidative damage.

Reproductive failure could be attributed to DNA damage. H_2O_2 can induce DNA single-strand breaks (20-24). DNA double-strand breaks due to H_2O_2 were observed in several studies (20, 23), but not in others (21, 24). DNA-protein cross-links are consistently absent from HX/XO- or H_2O_2-challenged cells (21, 24).

Table 2. The effect of HX/XO, H_2O_2 and TEMPO on •OH formation

Treatment	2,5-DHBA (nM)[a]
None	30.4±21.2
H_2O_2	216.8±54.7
H_2O_2 + 1 mM TEMPO	13.7±1.8
None	3.8±6.0
HX/XO	41.8±24.0
HX/XO + 1 mM TEMPO	< 1.56

[a] Mean ± SD of three independent determinations.

Increased rates of sister chromatid exchange have been reported (22). There is no agreement, however, on the possible relationship between any of these lesions and cell death.

Base modifications accompany oxidative stress to DNA *in vitro* and *in vivo* (11, 25, 26). This damage is repairable in most non-senescent cells and the modified bases appear in the incubation medium of cell cultures or the urine of whole organisms (11, 27). Oxidative damage to DNA and RNA can be measured as the formation of oxo^{8}dG and oxo^{8}G respectively. Cells exposed to H_2O_2 showed no significant increase in guanine hydroxylation products in cells (unrepaired nucleosides) or in the incubation medium (excised nucleosides or bases), Table 3. It is unlikely that DNA modifications are involved in the clonogenic failure.

While H_2O_2 rapidly equilibrates across cell membranes, the protection provided by the strictly extracellular CAT1 strongly suggests that the primary damage occurred at the cell surface. The ability of TEMPO to enter the cells did not confer a greater protective capacity than CAT1, suggesting that intracellular sites contribute minimally to the primary damage. The absence of nucleoside hydroxylation products also suggests that cytoplasmic sites are of little importance as primary targets of oxidative damage.

In our experiments, CHO cells exposed to low levels of oxidative stress are not killed. There is no microscopic evidence of apoptosis. They are interrupted in G_1 and G_2 of the cell cycle (manuscript in preparation). Several factors indicate that no major disruption occurred in membrane function and energy balance under low-level oxidative stress. LDH was not released from cells. The membranes were not rendered penetrable to the charged, small molecular weight probes CROX and CAT1. The ability of the cells to exclude trypan blue, which depends on both membrane integrity and a normal cellular energy charge, further testifies to the absence of overt damage to the membranes or to energy metabolism. There is no evidence that the oxidative damage is "global." It is quite likely that one or more sites at the cell surface are affected.

Pathways for the transduction of signals from the cell surface to the nucleus resulting in modification of transcription or in cell cycle arrest have been recently reviewed (28-30). The relevance of such a mechanism to oxidative stress is as yet unclear. H_2O_2 can activate protein kinase C (PKC) in endothelial cells (31). These authors showed that inhibition of PKC induction prevented the cellular effects of H_2O_2. Several nuclear responses induced by signal transduction from cell surface receptors are also induced by PKC activation (29). The induction of transcription factors by UV irradiation is triggered by a signal which is not of nuclear origin

Table 3. DNA and RNA damage in CHO cells under oxidative stress

	Degradation Products[a]			
	Experiment 1		Experiment 2	
Product	Untreated	H_2O_2-treated	Untreated	H_2O_2-treated
oxo8dG in cell DNA	100.0±16	117.0±9.6	100.0±34	114.0±20
oxo8dG in medium	100.0±8.3	50.0±8.3	100.0±21	2.3±0.0
oxo8G in medium	100.0±3.6	52.1±12	100.0±22	8.2±0.0
oxo8Gua in medium	100.0±1.4	154.0±64	100.0±12	9.3±2.3

[a] Results presented as normalized means ± standard deviation of representative experiments performed with duplicate (medium) or triplicate (cells) samples.

(32). It is quite possible that oxidant induced reproductive impairment is initiated by a surface event. We are currently investigating the nature of the message or the reaction product generated by the oxidative stress.

CHO cells subjected to oxidative stress had decreased clonogenic survival, without impairment of membrane integrity, disruption of cellular energy metabolism, or oxidation of DNA or RNA. Interference with cell proliferation in confluent human fibroblasts by H_2O_2 was recently observed (33). Chen and Ames saw no decrease in cell survival, but the H_2O_2-treated quiescent cells were unable to reenter the cell cycle in response to mitogenic signals. Instead, the fibroblasts entered a state resembling senescence. Our results show that actively dividing cells treated with low levels of H_2O_2 are arrested and appear to exit the cell cycle.

The ability of low-level oxidative stress to cause impairment of cell proliferation is quite different from the widely held concept of free radical damage as a "global" effect, causing disruptions throughout the cell. This rather subtle interference with cell function contrasts with the dramatic manifestations to which we have become accustomed, such as disruption of mitochondrial structure, fragmentation of the DNA or leakage of cytosolic components from the cell. The reproductive effects found in this study could mean that the involvement of free radicals in normal and pathological cell function is much more extensive and diverse than previously thought.

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