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CELL PROLIFERATION, REACTIVE OXYGEN AND CELLULAR GLUTATHIONE

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□ A variety of cellular activities, including metabolism, growth, and death, are regulated and modulated by the redox status of the environment. A biphasic effect has been demonstrated on cellular proliferation with reactive oxygen species (ROS)—especially hydrogen peroxide and superoxide—in which low levels (usually submicromolar concentrations) induce growth but higher concentrations (usually >10-30 micromolar) induce apoptosis or necrosis. This phenomenon has been demonstrated for primary, immortalized and transformed cell types. However, the mechanism of the proliferative response to low levels of ROS is not well understood. Much of the work examining the signal transduction by ROS, including H_2O_2, has been performed using doses in the lethal range. Although use of higher ROS doses have allowed the identification of important signal transduction pathways, these pathways may be activated by cells only in association with ROS-induced apoptosis and necrosis, and may not utilize the same pathways activated by lower doses of ROS associated with increased cell growth. Recent data has shown that low levels of exogenous H_2O_2 up-regulate intracellular glutathione and activate the DNA binding activity toward antioxidant response element. The modulation of the cellular redox environment, through the regulation of cellular glutathione levels, may be a part of the hormetic effect shown by ROS on cell growth.

Keywords: oxidative stress, antioxidant response element, MAPK, biphasic response, signal transduction

Abbreviations: AP-1, activator protein 1; ARE, antioxidant response element; BHK-21, baby hamster kidney fibroblasts; Cu/Zn-SOD, copper/zinc superoxide dismutase; FBS, fetal bovine serum; γGCS, gamma-glutamyl cyteine synthetase; MAPK, mitogen activated protein kinase; MnSOD, manganese superoxide dismutase; NAC, N-acetyl-L-cysteine; NADH, nicotinamide adenine dinucleotide; NAD(P)H, nicotinamide adenine dinucleotide phosphate; NF-κB, nuclear factor kappa B; NOX, NAD(P)H oxidase homolog; PAEC, pulmonary artery endothelial cells; ROS, reactive oxygen species; RPMEC, rat pulmonary microvascular endothelial cell; SMC, smooth muscle cells; SOD, superoxide dismutase; TGFβ1, transforming growth factor beta1.

INTRODUCTION

The evolution of mammalian cells in the oxidative atmosphere has resulted in development of sensitivity of many cellular functions to the oxidative/reductive status of both the extracellular and intracellular envi-

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ronments. The redox status of the intracellular milleu influences cellular activities including signal transduction, metabolism, growth, apoptosis and cellular systems involved in detoxification. Reactive oxygen species (ROS) including superoxide ($O_2^-\cdot$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO\cdot$) are known to inhibit activities of various biological molecules. More recently, low levels of ROS have been recognized to serve as second messengers for signal transduction (Halliwell, 1996; Suzuki *et al.*, 1997).

$O_2^-\cdot$ is generated via electron reduction of molecular oxygen O_2. Produced $O_2^-\cdot$ often undergoes disproportionation reactions in which one molecule of $O_2^-\cdot$ donates an electron to another, forming H_2O_2 and O_2 in a reaction named dismutation. The rate of dismutation reaction can be substantially increased by the enzyme superoxide dismutase (SOD), which occurs in either the copper/zinc (Cu/ZnSOD) or manganese (MnSOD) isoform. In addition to donating an electron to another $O_2^-\cdot$, $O_2^-\cdot$ can also donate an electron to ferric ion (Fe^{3+}) to form ferrous ion (Fe^{2+}), which in turn reduces H_2O_2 and causes an homolytic fission of the oxygen-oxygen bond to form a very potent oxidant $HO\cdot$ in the Fenton reaction. $HO\cdot$ in turn can react with virtually any orgainic compounds with very high kinetic rate constants, thus damaging a variety of biological molecules. To minimize the production of $HO\cdot$, H_2O_2 levels are tightly controlled by several enzymes, including catalase, glutathione peroxidase, thioredoxin and peroxiredoxins (Suzuki *et al.*, 1997; Veal *et al.*, 2004; Wood *et al.*, 2003; Zhao and Holmgren., 2002). Glutathione peroxidase utilizes reduced glutathione (GSH) to perform two electron reduction of H_2O_2 to produce H_2O. Oxidized glutathione can then be reduced via glutathione reductase.

Endogenous generation and exogenous application of ROS have been shown to directly activate specific signaling pathways. Cellular responses to large changes in the redox state of the environment are designed to maintain the cellular oxidative balance and avoid DNA, protein and lipid damage which could potentially lead to apoptosis or necrosis. However, in some cases, the cellular response to small changes in the ROS concentrations can have beneficial effects on cell growth and viability. This article summarizes work by our laboratory and others on the mechanisms of non-linear actions of ROS on cell growth.

## EFFECTS OF ROS ON GROWTH OF PRIMARY CULTURE, IMMORTALIZED AND TRANSFORMED CELLS

Burdon *et al.* (1989) demonstrated that low concentrations (10^{-6} to 10^{-8} M) of H_2O_2 and *t*-butyl hydroperoxide could stimulate growth in both primary fibroblast cultures and transformed baby hamster kidney fibroblasts (BHK-21) in the absence of serum (Burdon *et al.*, 1989). Higher concentration of these ROS caused reduced cell number, while even lower

concentrations had no effect on cell numbers, thus delineating a biphasic effect of the compounds (Burdon *et al.*, 1989; Burdon and Rice-Evans, 1989). Later studies showed that the biphasic effect of H_2O_2 on cell growth also occurred in cultures of untransformed and Ras-transformed fibroblasts, hepatoma cells, cultured smooth muscle cells, primary culture of pulmonary artery endothelial cells (PAEC), primary culture of rat microvascular endothelial cells (PMEC), and immortalized lung epithelial cells (A549) (Arnold *et al.*, 2001; Burdon, 1995; Burdon *et al.*, 1990; Day *et al.*, 2003; Gallagher *et al.*, 1993; Liu *et al.*, 2002). Dose-response H_2O_2 treatment of primary bovine PAEC (Fig 1A) and primary rat PMEC (Fig 1C) showed the existence of a biphasic curve, where 1 μM H_2O_2 increased cell number and higher levels (>10 μM) decreased cell number. Although treatment of PAEC with H_2O_2 alone induced an increase in cell number, the effect of low concentrations of H_2O_2 was greater in the presence of 2% FBS (Fig 1B) (Day *et al.*, 2003). The exact dosages of H_2O_2 resulting in cell growth is cell-type and cell density specific; some mammalian fibroblasts display growth in response to H_2O_2 at levels as high as 15 μM (Davies, 1999), but a biphasic effect is still observed.

$O_2^-\cdot$ has also been shown at low doses to induce growth in a variety of cell types including human and murine fibroblasts, human leukemia cells, human amnion cells, epithelial cells, and human smooth muscle cells (Burdon, 1995; Li *et al.*, 1997; Li *et al.*, 1999; Murrell *et al.*, 1990). Superoxide generated in these cell cultures using hypoxanthine with xanthine oxidase (≤1 μ unit xanthine oxidase/ml) induced proliferation that was suppressed by co-incubation of cells with superoxide dismutase (SOD) but not by co-incubation with catalase, showing that the growth-inducing species was in fact $O_2^-\cdot$ and not due to its reduction to H_2O_2 (Murrell *et al.*, 1990).

MULTIPLE OUTCOMES OF HIGH LEVELS OF ROS TREATMENT IN CULTURED CELLS

Investigation of the effects of higher levels of ROS on eukaryotic cells show the occurrence of temporary growth arrest, senescence, apoptosis, or necrosis (reviews, Boonstra and Post, 2004; Davies, 1999; Fiers *et al.*, 1999; Frippiat *et al.*, 2002; Remacle *et al.*, 1992, 1995). Treatment of fibroblasts with H_2O_2 showed that growth occurred between 3 to 15 μM. Temporary growth arrest occurred between 120 to 150 μM, associated with the expression of DNA repair and antioxidant enzymes. Permanent growth arrest, not associated with cell death, occurred between 250 to 400 μM. At treatments between 0.5 to 1 mM classical markers for apoptosis were observed, and necrosis occurred at levels above 5 mM (Davies, 1999). FACS and propidium iodide staining were used to show that concentrations of H_2O_2 from 30 μM–100 μM were associated with apoptotic cell death in primary PAEC; the lower level of H_2O_2 treatment resulting
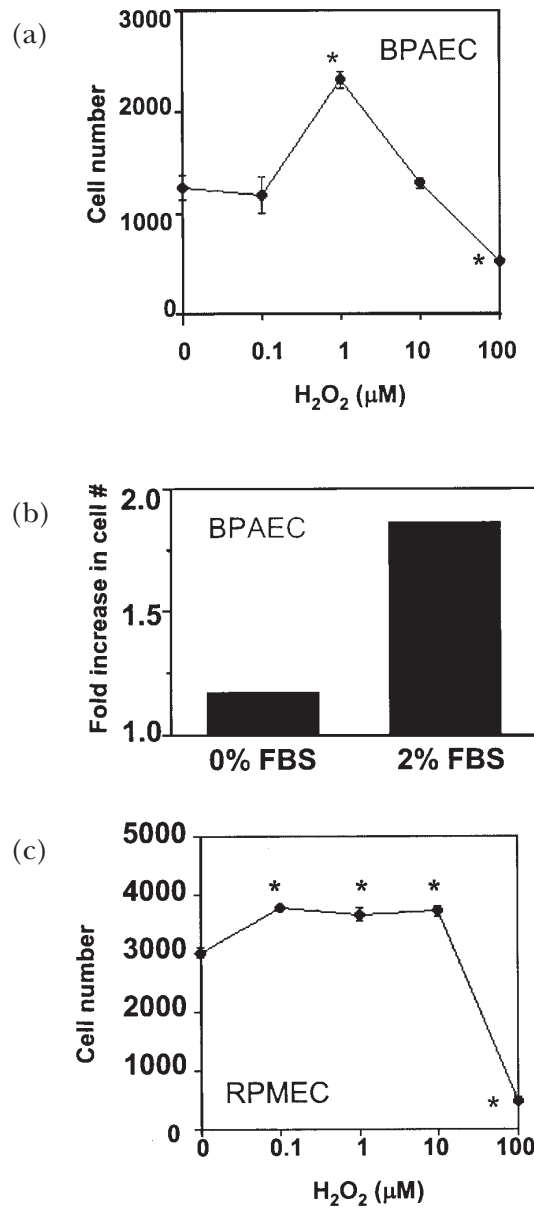
FIGURE 1 Effects of H_2O_2 on bovine PAEC and RPMEC growth. (A) Bovine PAEC (BPAEC) were treated with varied concentrations of H_2O_2 for 48 h in medium containing 2% FBS. (B) BPAEC were treated with 1 μM H_2O_2 for 48 h in medium containing 0 or 2% FBS. (C) RPMEC were treated with varied concentrations of H_2O_2 for 48 h in medium containing 2% FBS. The number of cells was determined by Coulter counting. Values represent means ± SE, n=3. *Values differ from the 0 μM H_2O_2 control at p<0.05. Work of Day *et al.* is being reproduced with permission (Day *et al.*, 2003).

in apoptosis suggests that these cells were more sensitive than fibroblasts to oxidative stress (Day *et al.*, 2003). In the case of exposure of a macrophage cell line to nitric oxide donors, both apoptosis and necrosis

were found; cellular apoptosis was attributed to the generation of H_2O_2 and caspase activation, while necrosis was attributed to reduced ATP generation and energy failure in conjunction with thiol depletion (Borutaite and Brown, 2003). The differentiation between the outcomes involving cellular senescence, apoptosis and necrosis appears to be cell-type and cell density specific, ROS species specific, and likely involves differential signal transduction and enzyme activation as well as differences in gene expression and/or DNA damage.

WHAT CONCENTRATIONS OF ROS ARE BIOLOGICALLY RELEVANT?

Production of ROS was initially investigated in neutrophils, which generate large quantities of ROS for the destruction of bacteria, infected cells and abnormal cells. However, the production of lower levels of ROS has also been examined in non-leukocytes, and has been demonstrated to occur to varying degrees and in response to a variety of stimuli in all cell types examined to date. The mechanism of generation of ROS by non-phagocytic cell types involves the assembly of NADH or NAD(P)H oxidases at the cell membrane (Griendling and Harrison, 1999; Thannickal *et al.*, 2000). The phagocytic NAD(P)H oxidase has been the most studied, but oxidase complexes containing gp91 homologs (NOX-1, NOX-3 or NOX-4) have been identified in other cell types, including endothelial and epithelial cells, fibroblasts, osteoclasts and vascular smooth muscle cells (Babior, 2000; Bayraktutan *et al.*, 2000; Chamseddine and Miller, 2003; Murphy *et al.*, 2000; Perner *et al.*, 2003; Yang *et al.*, 2001b; Zafari *et al.*, 1998). These NADH and NAD(P)H oxidase systems have been shown to contain a gp91 homolog (NOX), p67 phox, p22 phox and p47 phox (Hohler *et al.*, 2000; Jones *et al.*, 1996; Parinandi *et al.*, 2003; Sorescu and Griendling, 2002; Zafari *et al.*, 1998). Several of the Ras-related small GTP-binding proteins, including Ras and Rac1, have also been implicated in the generation of ROS by the nonphagocytic oxidases (Joneson and Bar-Sagi, 1998; Sundaresan *et al.*, 1996; Thannickal *et al.*, 2000).

Measurements of endogenous generation of active oxygen species have been performed on primary, immortalized and transformed eukaryotic cells. In 1991, Kinnula *et al.* examined the production of extracellular H_2O_2 by freshly isolated alveolar epithelial Type II cells, unstimulated alveolar macrophages and primary endothelial cells (Kinnula *et al.*, 1991). Results showed that macrophages produced the highest level of extracellular peroxide (3.1 ± 0.09 nmol·min^{-1}·mg protein^{-1}, equivalent to $\sim0.1\times10^6$ cells), followed by Type II alveolar cells (0.7 ± 0.07 nmol·min^{-1}·mg protein^{-1}, $\sim0.5\times10^6$ cells) and then by vascular endothelial cells (0.4 to 0.06 ± 0.005 nmol·min^{-1}·mg protein^{-1}, $\sim3\times10^6$ cells) (Kinnula *et al.*, 1991; Zulueta *et al.*, 1995). Extracellular superoxide production has also been measured in non-leukocytes. From data available, fibroblasts appear to release the highest levels of O_2^-· (~1 nmol·h^{-1}·1×10^5 cells)

(Murrell *et al.*, 1990), followed by endothelial cells (~0.6 nmol·h^{-1}·1×10^5 cells) (Weening *et al.*, 1975), and then unstimulated granulocytes (~0.4 nmol·h^{-1}·1×10^5 cells) (Matsubara and Ziff, 1986).

The level of ROS produced by cells can be regulated by the redox state of the environment. Exposure of cells to hypoxia increased the release of H$_2$O$_2$ by about 25–30% (Kinnula *et al.*, 1991; Parinandi *et al.*, 2003). Experiments by Parinandi *et al.* (2003) showed that exposure of PAECs to hyperoxia induced ~2.5-fold increase in ROS production within 1 h (Parinandi *et al.*, 2003). These studies suggest that cells adjust their endogenous ROS production in response to oxidative stress.

The observations of cellular generation of ROS, and the speculation that these molecules act as second messengers, led to the hypothesis that there is a balance between cell growth and endogenous ROS production (Burdon, 1995). Burdon *et al.* (1995) examined endogenous intracellular H$_2$O$_2$ production by immortalized BHK-21 fibroblast cells, and found that levels of H$_2$O$_2$ production decreased with increasing time in culture and confluence (from ~0.15 nmol/10^6 cells to ~0.015 nmol/10^6 cells after 72 h in culture) (Burdon *et al.*, 1995). Sub-confluent transformed epithelial cells also produced significantly more ROS than confluent cells (Perner *et al.*, 2003). Our laboratory has shown that the level of extracellularly generated H$_2$O$_2$ production by primary PAEC and primary human embryonic fibroblasts (IMR90) also decreases with incubation in serum-free medium (Fig. 2). These results show that decreased ROS production correlates with decreased rates of growth.

Interestingly, some tumor cell lines constitutively produce high levels of extracellular ROS, which are associated with their increased proliferation (Perner *et al.*, 2003; Pustovidko *et al.*, 2000; Schimmel and Bauer, 2002; Suh *et al.*, 1999). Exogenous expression of NOX1 in NIH3T3 fibroblasts caused increased cell growth, abnormal cellular morphology and the ability to form tumors in athymic mice (Arnold *et al.*, 2001). In these experiments, 10-fold over-expression of NOX-1 in NIH3T3 fibroblasts induced increased growth and transformation with only <2-fold increase in extracellular O$_2^-$· generation, showing that high levels of ROS are not required to achieve a growth or transformation. Co-expression of catalase reversed the transformed phenotype, indicating that, in this case, H$_2$O$_2$ was the growth-promoting species (Arnold *et al.*, 2001).

SIGNAL TRANSDUCTION BY HIGH VERSUS LOW DOSES OF ROS

Studies of ROS-induced signal transduction have mostly focused on the use of large doses of ROS and their cellular responses. These studies have been instrumental in the identification of signaling pathways and specific molecules responsive to ROS. Exogenously added ROS at high levels (>10 μM) directly inactivate protein phosphatases, thus shifting the

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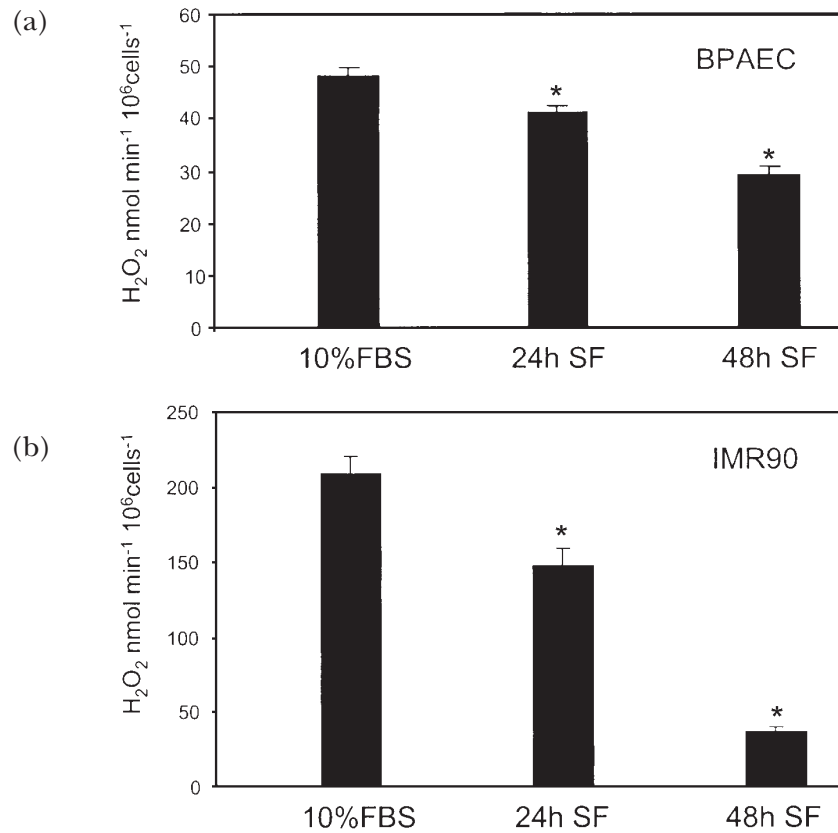
FIGURE 2 Effect of growth arrest on endogenous production of extracellular H_2O_2 by bovine PAEC and IMR90. Bovine PAEC (BPAEC) (A) or IMR90 human lung fibroblasts (B) were subcultured at 4×10^4 cells/35 mm dish in RPMI supplemented with 10% FBS. 24 h and 48 h after plating, medium was replaced with serum free (SF) RPMI. Extracellular H_2O_2 was determined as described previously (Thannickal *et al.*, 1993), and normalized to cell numbers. Briefly, cells were washed with Hanks' buffered saline, without phenol red, pH 7.4. Cells were then placed in Hanks saline containing 1mM HEPES, 100 μM homovanillic acid, 5 units/ml horseradish peroxidase, type IV. The conditioned medium was collected after 1 h, and the pH was adjusted to 10.0 with 0.1 M glycine-NaOH buffer. Fluorescence was measured at excitation and emission wavelengths of 321nm and 421nm, respectively. Control samples were made containing the reaction mixture alone, and incubated in the absence of cells to correct for spontaneous dimerization of homovanillic acid. A standard curve was generated using known concentrations of H_2O_2 incubated with the reaction solution. Values represent means ± SD, n=3. * Values differ from 10% FBS at p<0.05.

balance of kinase/phosphatase activity in the cell toward increased phosphorylation events, and resulting in the activation of specific signaling pathways (Guy *et al.*, 1993; Rhee *et al.*, 2000; Suzuki *et al.*, 1997; Thannickal and Fanburg, 2000). Additionally, ROS are believed to directly activate some kinases, including protein kinase C and MAP kinase (Gopalakrishna and Anderson, 1989; Suzuki *et al.*, 1997; Thannickal and Fanburg, 2000). Recently, it was shown that the heterotrimeric proteins G_i and G_o can also be directly activated by ROS (Nishida *et al.*, 2002).

Low oxygen environments induce both the stability and activity of the hypoxia-inducible factors (HIFs) which regulate transcription of a variety of growth factors and glycolytic enzymes for anaerobic energy production (Hoshikawa *et al.*, 2003; Lando *et al.*, 2002). Highly oxidative environments also result in the induction of detoxification enzymes, via transcription factors including the antioxidant response element (ARE) binding proteins (such as members of the Nrf family), activator protein 1 (AP-1), and NF-κB (Griffith, 1999; Thannickal and Fanburg, 2000; Wild and Mulcahy, 2000).

In contrast with the numbers of studies performed using toxic levels of ROS (usually >30 μM), relatively few studies have been performed to examine signal transduction by lower concentrations of ROS. As stated above, 10-fold over-expression of NOX-1 in NIH3T3 fibroblasts induced cellular growth and transformation with <2-fold increase in extracellular $O_2^-\cdot$ generation; growth was associated with increased expression of a variety of genes related to cell cycle regulation and cancer but not to oxidative stress (Arnold *et al.*, 2001). A study by Cumming, *et al.*, examined global changes in disulfide bond formation following ROS exposure (Cumming *et al.*, 2004). The results showed treatment of HT22 cells to 10 μM H_2O_2 affected several lower molecular weight proteins but had little effect on the disulfide bonding of proteins in the 40–150-kDa range. However, a number of disulfide bonded proteins in the 40–150-kDa range were affected by 150 or 400 μM H_2O_2 treatment. The results suggest that uniquely disulfide bonded proteins which occur at low levels of H_2O_2 exposure may be involved in the mitogenic effects of low oxidant concentrations on cultured cells. In contrast, proteins that are disulfide-linked at intermediate H_2O_2 concentrations may participate in the adaptive response or growth arrest. These findings support the hypothesis that growth-inducing levels of ROS may not signal via the same pathways as those found to be activated by treating cells with high doses of ROS associated with growth arrest or apoptosis.

Extracellular ligands including fibroblast growth factor, platelet-derived growth factor, endothelin, interferon γ, tumor necrosis factor α, and members of the interleukin family have been shown to induce the production of low levels of intracellular ROS (Finkel, 2000; Finkel, 2001; Thannickal and Fanburg, 2000). Antioxidants or antioxidant enzymes have been used to inhibit signal transduction downstream of these factors as a method of identifying specific proteins responsive to ROS second messengers. Such experiments have been used to prove ROS inactivation of protein phosphatases, as well as activation of signal transduction proteins such as MAP kinases (including p38, p42/p44 and jun kinase), phospholipase A2, and the transcription factors NF-κB, Egr-1 and c-Fos (Finkel, 2001; Thannickal and Fanburg, 2000; Xu *et al.*, 2002). In many cases the elimination of ROS generation abrogates downstream biological responses, including cell growth (Xu *et al.*, 2002).

Changes in the redox environment can also be attained by over-expression or inhibition of anti-oxidant enzymes, with subsequent effects on cell growth/apoptosis and signaling. Adenoviral over-expression of catalase (50-100-fold excess) causes decreased proliferation and DNA synthesis and a rise in apoptosis in vascular smooth muscle cells (Brown *et al.*, 1999). Vector expression of Cu/Zn-SOD in microglial cells prevents cellular activation by LPS; here, the inhibition is hypothesized to occur due to scavenging of $O_2^-\cdot$, a second messenger required for LPS signaling (Chang *et al.*, 2001).

As discussed above, many cancer cell lines produce increased levels of ROS; some of these transformed cells also express higher levels of SOD and/or catalase, possibly to catalyze the breakdown of excess superoxide and H_2O_2 (Chung-man Ho *et al.*, 2001; Grigolo *et al.*, 1998; Hur *et al.*, 2003; Janssen *et al.*, 1999; Malafa *et al.*, 2000; Palazzotti *et al.*, 1999; Suresh *et al.*, 2003). This adaptive effect protects the cells from the higher level of oxidant stress, and also often results in their resistance to anti-cancer treatments (Chung-man Ho *et al.*, 2001; Hur *et al.*, 2003; Suresh *et al.*, 2003; Yang *et al.*, 2001a). Even in these cases, with up-regulation of both ROS production and antioxidant enzymes, the overall redox balance is maintained to favor growth-promoting signal transduction by the ROS. In ovarian cancer cells, pancreatic adenocarcinoma and prostate tumor cells, exogenous over-expression of MnSOD led to reduced growth rates (Cullen *et al.*, 2003; Plymate *et al.*, 2003; Takada *et al.*, 2002). In some cases, scavenging of H_2O_2 inhibited the proliferation of transformed fibroblasts and reverted their phenotype (Arnold *et al.*, 2001; Preston *et al.*, 2001).

GLUTATHIONE AND ANTIOXIDANT ENZYME LEVELS: REDOX BALANCE FOR GROWTH, QUIESCENCE OR APOPTOSIS

The primary defenses of eukaryotic cells against oxidation include antioxidant enzymes SOD and catalase, and GSH-utilizing mechanisms. GSH, which is present in millimolar concentrations in cells, acts to prevent the oxidation of other cellular molecules and functions in Phase II detoxification of xenobiotics (Griffith, 1999; Wild and Mulcahy, 2000). The ratio of intracellular thiol reductants and ROS appears to play a pivotal role in determining whether cells undergo growth or apoptosis (Burdon, 1995). Imbalance of the redox status, either by high levels of oxidation or increase of reducing agents, favors decreased growth and increased apoptosis.

The level of cellular GSH is responsive to the redox state of the cellular environment as well as the growth state of the cell (Burdon *et al.*, 1994; Day *et al.*, 2003; Griffith, 1999; Wild and Mulcahy, 2000). GSH is increased in response to endogenous and exogenously added active oxygen species (Burdon *et al.*, 1994; Day *et al.*, 2003; Day *et al.*, 2002). The direct response of cells to an increase in the oxidation state of the environment occurs through the transcriptional regulation of genes that reg-

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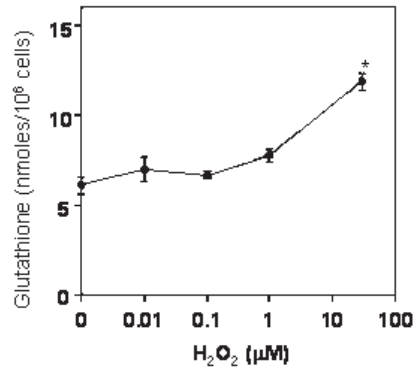
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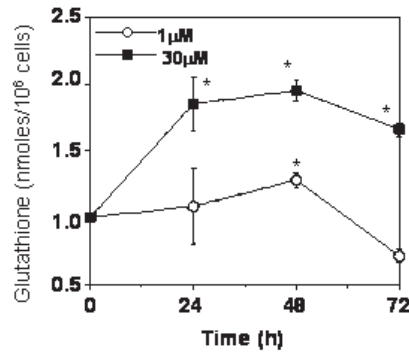
FIGURE 3 Effects of H_2O_2 on cellular glutathione content. (A) Bovine PAEC (BPAEC) were treated with varied concentrations of H_2O_2 for 48 h in medium containing 2% FBS. Levels of total glutathione were determined as previously described (Day *et al.*, 2003). Briefly, cells were detached from dishes using trypsin/EDTA to produce a cell suspension. A portion of the suspension was used to obtain a cell count while the remaining cells were treated with 1% perchloric acid. The perchloric acid supernatants were sonicated on ice and adjusted to pH 7. Total glutathione was measured in a kinetic assay using glutathione reductase, β-NADPH and gluthathione disulfide. The reduction of 5,5´-dithiobis(2-nitrobenzoic acid) was followed spectrophotometrically at 412 nm. Values represent means ± SD, n=3; experiments were performed at least 3 times. * Values differ from 0 μM H_2O_2 at p<0.05. (B) Bovine PAEC were treated with 1 or 30 μM H_2O_2 for the durations indicated. The graph shows means ± SE, n=3, of fold increase in glutathione. *Values differ from 0 time point at p<0.05. Work is being reproduced with permission (Day *et al.*, 2003).

ulate glutathione synthesis as well as uptake of GSH precursors (Day *et al.*, 2003; Day *et al.*, 2002; Deneke and Fanburg, 1989; Deneke *et al.*, 1987). Our laboratory has shown that sub-lethal doses of H_2O_2, like toxic levels of H_2O_2, up-regulate cellular glutathione levels although the time course is delayed by ~24 h (Fig. 3) (Day *et al.*, 2003). Although 1 μM H_2O_2 does
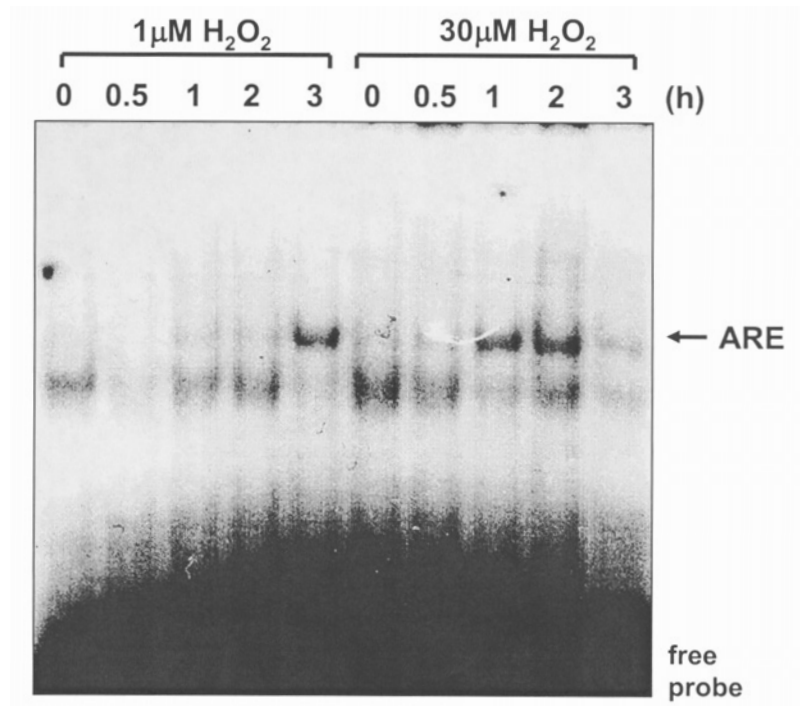
FIGURE 4 Effects of H_2O_2 on ARE binding. Bovine PAEC were treated with the indicated concentrations of H_2O_2 for 0.5 to 3 h. Nuclear extracts were prepared and DNA-binding activity to an ARE consensus oligo was monitored by EMSA. To prepare nuclear extracts, cells were washed in PBS and incubated in 10 mM Hepes (pH 7.8), 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM NaF, 0.1 mM sodium orthovanadate, and 1 mM tetrasodiumphyrphosphate for 15 min at 4°C. IGEPAL CA-630 was then added at a final concentration of 0.6% (v/v). Samples centrifuged and pelleted nuclei were resuspended in extraction buffer (50 mM Hepes (pH 7.8), 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM NaF, 0.1 mM sodium orthovanadate, and 1 mM tetrasodiumphyrphosphate, and 1% glycerol) and mixed vigorously for 20 min, 4°C, and centrifuged for 5 min. Supernatants were harvested and protein concentrations were determined. To perform EMSA binding reaction, mixtures containing 2 μg nuclear extract protein were incubated with 1 μg poly(dI-dC)·poly(dI-dC) and ^{32}P-labeled double-stranded oligonucleotide containing a consensus sequence for ARE in 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol (v/v), and 20 mM Tris-HCl (pH 7.5) for 20 min at 25°C. Electrophoresis of samples through a native 6% polyacrylramide gel was followed by autoradiography. This work is being reproduced with permission (Day *et al.*, 2003). The primary species binding to the ARE in PAEC was previously identified to be Nrf-2 (Day *et al.*, 2002).

not activate p42/p44 MAP kinase or NF-κB in PAEC, this low concentration of H_2O_2 does activate the binding of other transcription factors to the antioxidant response element (ARE), such as Nrf2, as determined by band shift assays (Fig. 4) (Day *et al.*, 2002; Day *et al.* 2003) and by Nrf2 expression (Pi *et al.*, 2003). Interestingly, there is a 2 h delay in the activation of ARE-binding proteins by 1 μM compared with the 30 μM dose. The ARE element in the promoter of γ-glutamylcysteine synthetase (γGCS) has been shown to be required for the regulation of this gene in
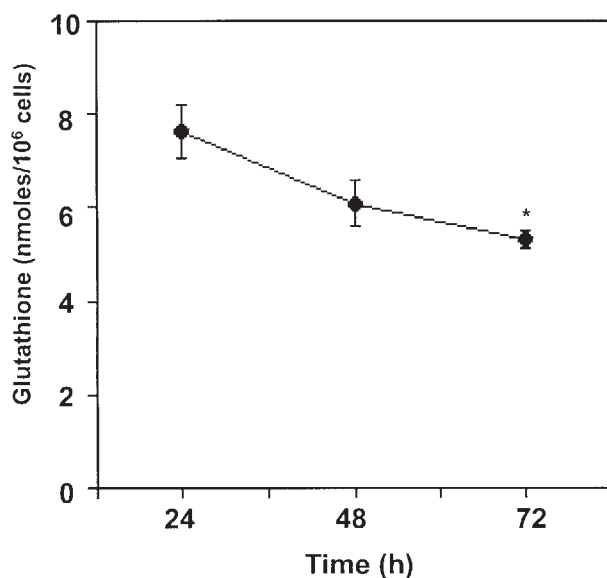
FIGURE 5 Effects of cell density on glutathione content. Bovine PAEC were grown for the durations indicating after plating at 4×10^4 cells/35 mm dish in RPMI supplemented with 10% FBS. Total cellular glutathione was measured as stated in Figure 3. Values represent means ± SE, n=3. *Values differ from the 24 h value at $p<0.05$. Work is being reproduced with permission (Day *et al.*, 2003).

response to some xenobiotic agents (Wild and Mulcahy, 2000). Because γGCS controls a key step in the synthesis of glutathione, regulation of this enzyme is of interest for understanding cellular response to ROS.

Glutathione imbalances in the cell have been linked to specific changes in signal transduction downstream of growth factors or cytokines, ultimately leading to changes in the overall outcome of signaling. *N*-acetyl-L-cysteine (NAC, a glutathione precursor which increases intracellular levels of glutathione), inhibits growth induced by endothelin-1 in smooth muscle cells (Kyaw *et al.*, 2002); this affect was attributed to the reduction of second-messenger ROS generation by endothelin-1, leading to the failure to activate specific MAP kinases. In lymphocytes, drastically decreasing cytoplasmic levels of glutathione by treatment with buthiomine sulfoximine causes increased tyrosine phosphorylation in response to tumor necrosis factor α (Anderson *et al.*, 1994; Staal *et al.*, 1994). Levels of oxidized/reduced glutathione may also play a direct role in signal transduction, especially with regard to Ca^{2+} release from intracellular stores and cellular response to oxidative stress (Marcho *et al.*, 1991; Suzuki *et al.*, 1998). In many cases, an increased oxidative environment appears to favor increased kinase activity, while an increased reductive environment often correlates with suppressed kinase activation (Guy *et al.*, 1993; Suzuki *et al.*, 1997), although the overall balance remains critical for the proliferative outcome.
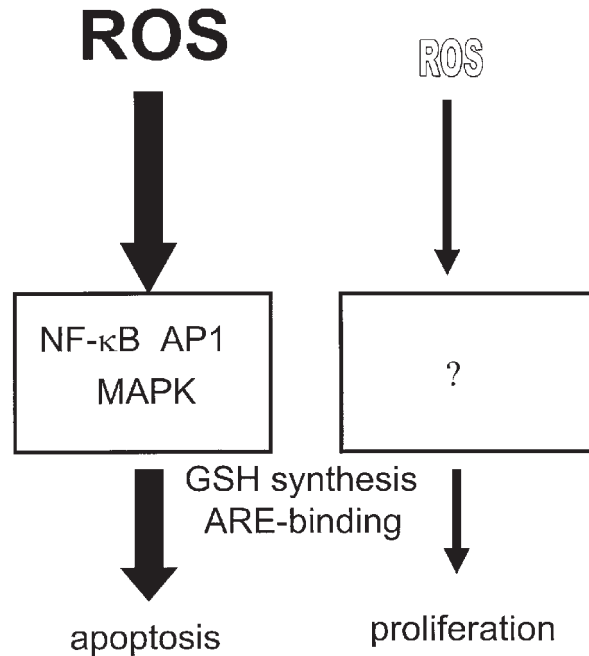
FIGURE 6 Scheme of biphasic ROS signal transduction. High levels of ROS (left side) induce activation of multiple MAP kinases and activate transcription factors including AP-1 and NF-κB; these pathways lead to apoptosis or necrosis. Low doses of ROS (right side) cause cell proliferation, but the signal transduction pathways involved are unknown. Both high and low doses of ROS up-regulate cellular glutathione and activate DNA-binding to the ARE element.

Experiments examining glutathione levels in growing versus growth arrested cells show that glutathione levels become reduced as cells approach quiescence (Fig. 5) (Burdon *et al.*, 1994; Day *et al.*, 2003); the reduction of endogenous GSH production occurs at the same time that cells are producing reduced levels of extracellular ROS, again suggesting that a cellular balance is being maintained (compare Fig. 2). Endothelial cell growth inhibition by treatment with transforming growth factor beta1 (TGFβ1) is associated with both decreased cellular glutathione and increased extracellular production of H_2O_2 (Das *et al.*, 1992; Thannickal *et al.*, 1993; White *et al.*, 1999). TGFβ-associated antiproliferative effects were found to be modulated by simultaneous treatment of PAEC with cysteine, cystine or NAC, precursors of glutathione synthesis (Das *et al.*, 1992; White *et al.*, 1999), again demonstrating that a balance between ROS and GSH can determine biological outcome of oxidative stress.

CONCLUSIONS

The response of eukaryotic cells to active oxygen species can induce proliferation, growth arrest, apoptosis, or necrosis, depending upon the

dose of ROS exposure. Our results show that in PAEC, 0.1 to 1.0 μM H_2O_2 causes growth, ~30 to 100 μM induces apoptosis, while higher levels may induce necrosis (Fig 6). The outcome of exposure to oxidative stress can vary with cell density and is cell type-specific, with some cell types showing a higher tolerance toward the apoptotic effects. The biphasic effect of ROS treatment on eukaryotic cells has been hypothesized to arise from the potential disruption in the balance in the redox environment of the intracellular milieu. Oxidative species function as second messenger molecules downstream of a number of growth-promoting factors, but ROS are also highly reactive and can cause damage to cellular proteins, lipids and DNA. Therefore, a delicate balance exists between the useful function of these molecules versus their destructive nature. While a number of signal transduction pathways have been identified for high doses of ROS in cells, very little is known about signal transduction by low, growth promoting levels of ROS. High dose ROS signaling is likely related to cellular apoptosis or necrosis, suggesting that these pathways may not be relevant for understanding the mechanisms involved for the proliferative response of cells to micromolar and submicromolar levels of ROS.

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