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Regulation of Glutathione by Oxidative Stress in Bovine Pulmonary Artery Endothelial Cells

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ABSTRACT

Glutathione plays important roles as an intracellular antioxidant and in the maintenance of cellular thiol–disulfide balance. In addition, glutathione may regulate cell growth signaling induced by oxidative stress. We previously reported that cellular glutathione is up-regulated by bleomycin in bovine pulmonary artery endothelial cells. The present study examined effects of hydrogen peroxide (H_2O_2) on cell growth and glutathione levels. Exogenous addition of H_2O_2 induced biphasic effects on cell growth; 1 μ*M* was stimulatory and >10 μ*M* was inhibitory. However, both growth-promoting and inhibitory levels of H_2O_2 increased cellular glutathione levels. Whereas 1 μ*M* H_2O_2 moderately but significantly increased glutathione, 30 μ*M* caused a more substantial increase. Like bleomycin, both concentrations of H_2O_2 activated DNA binding of antioxidant response element (ARE), a regulatory element in the promoter of the γ-glutamylcysteine synthetase heavy chain subunit, a key regulator of glutathione synthesis. However, only high concentrations of H_2O_2 activated p44/42 mitogen-activated protein (MAP) kinase. Thus, cellular glutathione is up-regulated by H_2O_2, perhaps via activating ARE-binding factors in a mechanism independent of MAP kinase. H_2O_2-mediated increase in glutathione and activation of ARE binding may play important roles in growth and death of pulmonary artery endothelial cells. *Antioxid. Redox Signal.* 5, 699–704.

INTRODUCTION

REACTIVE OXYGEN SPECIES, such as superoxide anion radicals, hydrogen peroxide (H_2O_2), and hydroxyl radicals, mediate various pathological conditions, including those affecting the lung. Glutathione plays important roles as an intracellular antioxidant and in the maintenance of cellular thiol–disulfide balance (12). Glutathione is regulated by stress and xenobiotics downstream of nuclear factor-κB (NF-κB) and antioxidant response element (ARE)-binding transcription factors (11, 13, 14). In bovine pulmonary artery endothelial cells (BPAEC), we have recently shown that the cellular levels of glutathione are up-regulated by oxidative stress induced by bleomycin (11). Thus, oxidative stress-responsive signal transduction pathways regulate glutathione expression in mechanisms believed to have evolved to protect cells from oxidant damage (14).

Although oxidants, such as H_2O_2 and superoxide, have often been examined at high concentrations, especially for their role in the induction of cellular damage, it has become clear that many oxidants, in lower doses, can promote cell growth. H_2O_2 may elicit either growth arrest or promotion of cell growth, depending on the cell type (2). In various cell lines, including BHK-21 baby hamster kidney fibroblasts, increased endogenous cellular generation of H_2O_2 is correlated with cellular proliferation (5, 6). Furthermore, the exogenous addition of low concentrations of H_2O_2 (<10 μ*M*) stimulates cell growth (4), and greater levels cause cell death in BHK-21 cells (6, 7). Interestingly, in these cells, growth-promoting exogenous H_2O_2 causes 30% reduction of the levels of cellular glutathione (5). Burdon (3) proposed that the primary growth-inducing mechanism of exogenously added low levels of H_2O_2 is not the direct activation of proliferation-associated signal transduction molecules, but rather the reduction of

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cellular glutathione levels. These results suggest complex relationships between cell growth, H_2O_2, and glutathione.

Our laboratory has shown that H_2O_2 is released from BPAEC constitutively in response to transforming growth factor-β1 (TGFβ) (16) and in response to hypoxia–reoxygenation (18). A ~60–70% reduction in cellular glutathione levels is associated with growth arrest by TGFβ in these cells (8, 17). The antiproliferative effect of TGFβ can be ameliorated by the addition of cysteine, cystine, and *N*-acetylcysteine with elevation of cellular glutathione (8), suggesting that oxidative stress induces growth arrest signals by decreasing cellular glutathione in these cells.

The present study investigated the effects of exogenous H_2O_2 on cell growth and glutathione levels in low-passage BPAEC. We found that low concentrations of H_2O_2 (1 μ*M*) stimulated and higher levels (>30 μ*M*) inhibited cell growth. Growth-promoting- H_2O_2 slightly and growth-inhibiting H_2O_2 substantially increased cellular glutathione in these cells. The mechanism of glutathione induction involves factors that bind to ARE as both concentrations of H_2O_2 activated the DNA-binding activity toward ARE.

MATERIALS AND METHODS

Cell culture

Primary BPAEC were either obtained from freshly slaughtered calves as described previously (9, 15) or purchased from Cell Applications, Inc. (San Diego, CA, U.S.A.). Passage 3–8 cells were used for all experiments. BPAEC were cultured in RPMI 1640 with antibiotics (penicillin and streptomycin), fungisone, and 10% fetal bovine serum (FBS). Rat pulmonary microvascular endothelial cells (RPMEC) were cultured as previously described (11). RPMEC were grown in RPMI 1640 with penicillin, streptomycin, fungisone, and 10% FBS. The pulmonary epithelial cell line A549 was maintained in Dulbecco's modified Eagle medium, with penicillin, streptomycin, fungisone, and 10% FBS. Cells were grown in 5% CO_2 at 37°C in a humidified atmosphere.

Determination of total glutathione

Cell counts and glutathione assays were performed as previously described (11). In brief, culture dishes were rinsed twice with phosphate-buffered saline at 25°C and incubated for 3–5 min with 1.0 ml of trypsin-EDTA. The cells were rapidly suspended and placed on ice. A 0.1-ml amount of the cellular suspension was removed, diluted, and counted using a ZM Coulter Counter (Coulter Electronics, Hialeah, FL, U.S.A.). An 0.8-ml amount of the remaining suspension was treated with 0.1 ml of 1% perchloric acid. Supernatants were aliquoted and frozen at −20°C for glutathione assays. Frozen perchloric acid-treated supernatants were thawed on ice and sonicated for 10 s on ice. The pH was adjusted to 7.0 with 0.3 *M* potassium hydroxide–3-(*N*-morpholino)propanesulfonic acid (MOPS). The sonicate was then centrifuged at 14,000 *g* at 4°C for 20 min. The supernatant was assayed for total cellular glutathione by the Tietze method (1). The sum of the oxidized and reduced forms of glutathione was determined using a kinetic assay in which reduced glutathione or glutathione disulfide plus glutathione reductase reduces 5,5′-dithiobis(2-nitrobenzoic acid) to form 5-thio-2-nitrobenzoate (TNB). The formation of TNB was followed spectrophotometrically at 412 nm. Each assay was individually calibrated with standard glutathione, and the concentration of each sample was adjusted by dilution to ensure that the reaction rate was on the linear portion of the standard curve. Cellular glutathione levels were expressed as nanomoles per 10^6 cells. For the assay, Brewers Yeast glutathione reductase, β-NADPH, and glutathione disulfide were obtained from Sigma (St. Louis, MO, U.S.A.).

Western blot analysis

To prepare lysates, cells were washed in phosphate-buffered saline and solubilized with 50 m*M* HEPES solution (pH 7.4) containing 1% (vol/vol) Triton X-100, 4 m*M* EDTA, 1 m*M* sodium fluoride, 0.1 m*M* sodium orthovanadate, 1 m*M* tetrasodium pyrophosphate, 2 m*M* phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Following centrifugation in a microfuge, protein concentrations in the supernatant were determined. Cell lysates (10 μg of protein) were electrophoresed through reducing (5% β-mercaptoethanol) sodium dodecyl sulfate polyacrylamide gels (10%) and electroblotted onto nitrocellulose membranes using a Bio-Rad Trans-Blot SD Semi-Dry Transfer Cell. After the transfer, the membranes were blocked and incubated with the polyclonal IgG for phospho-specific p44/42 mitogen-activated protein (MAP) kinase (Cell Signaling, Beverly, MA, U.S.A.). Levels of proteins and phospho-proteins were detected with horseradish peroxidase-linked secondary antibodies and the ECL System (Amersham Life Science, Arlington Heights, IL, U.S.A.).

Electrophoretic mobility shift assays (EMSA)

Procedures for nuclear extraction and EMSA have been described previously (11). The binding reaction mixtures contained 5 μg of protein of nuclear extract, 1 μg of poly(dI-dC)·poly(dI-dC), and ^{32}P-labeled double-stranded oligonucleotide containing ARE sequence (5′-TCA CAG TGA CTC AGC AGA ATC-3′) in 100 m*M* NaCl, 1 m*M* EDTA, 1 m*M* dithiothreitol, 10% (vol/vol) glycerol, and 20 m*M* Tris-HCl (pH 7.5).

Statistical analysis

Means ± SE were calculated, and statistically significant differences between two groups were determined by the Student's *t* test at $p < 0.05$

RESULTS

Effects of H_2O_2 on cell growth

Treatment of BPAEC with H_2O_2 (0–100 μ*M*) for 48 h resulted in dose-dependent, biphasic changes in cell number. A low dose of H_2O_2 (1 μ*M*) increased the cell number, whereas high levels (>10 μ*M*) caused a decrease in cell number (Fig. 1A). Apoptotic cell death was also observed with high

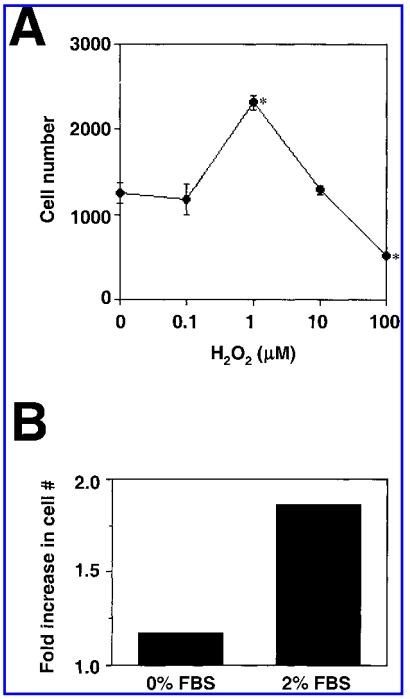

FIG. 1. Effects of H_2O_2 on BPAEC growth. (**A**) BPAEC were treated with varied concentrations of H_2O_2 for 48 h in medium containing 2% FBS. The number of cells was determined by the Coulter counting. Values represent means ± SE. *Values different from the 0 μ*M* H_2O_2 value at $p < 0.05$. (**B**) BPAEC were treated with 1 μ*M* H_2O_2 for 48 h in medium containing 0 or 2% FBS.

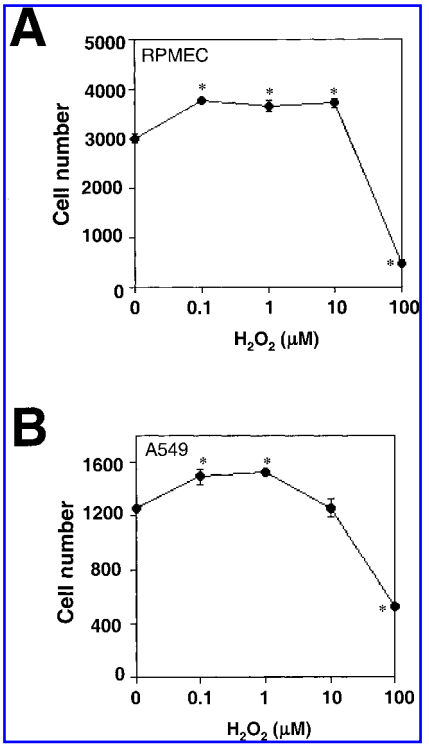

FIG. 2. Effects of H_2O_2 on growth of RPMEC and A549 cells. RPMEC (**A**) or A549 cells (**B**) were treated with varied concentrations of H_2O_2 for 48 h in medium containing 2% FBS. The number of cells was determined by the Coulter counting. Values represent means ± SE. *Values different from the 0 μ*M* H_2O_2 value at $p < 0.05$.

levels of H_2O_2 as monitored by FACS and propidium iodide staining (data not shown). The growth-promoting effects of 1 μ*M* H_2O_2, which occur in 2% FBS-containing medium, were not observed when cells were in serum-free medium (Fig. 1B). Thus, subsequent experiments were performed under conditions with 2% FBS unless otherwise noted.

To determine whether these H_2O_2-mediated biphasic changes in BPAEC number also occur in other lung-derived cells, the effects of H_2O_2 were also examined in two cell lines: RPMEC and A549 lung epithelial cells. We found that similar biphasic effects of H_2O_2 were also observed in RPMEC (Fig. 2A) and A549 cells (Fig. 2B), indicating that these biological events are not restricted to BPAEC.

Effects of H_2O_2 on cellular glutathione

To establish the relationship between BPAEC growth and cellular glutathione content, the levels of total (reduced and oxidized) glutathione were monitored. Treatment of cells with H_2O_2 for 48 h caused a dose-dependent increase in glutathione levels (Fig. 3A). Treatment of cells with growth-promoting, low levels (1 μ*M*) of H_2O_2 for 48 h slightly, but significantly, increased cellular glutathione levels from 6.1 ± 0.5 to 7.8 ± 0.3 nmol/10^6 cells ($p < 0.05$). At 30 μ*M* H_2O_2, which caused apoptosis, cellular glutathione was increased more substantially to 11.9 ± 0.5 nmol/10^6 cells. The time-course study showed that the increase in glutathione levels by 1 or 30 μ*M* H_2O_2 peaked at 48 h and subsequently declined by 72 h (Fig. 3B).

Effects of cell density on cellular glutathione

To determine the effects of proliferative and arrest status of the cells on glutathione levels, the total glutathione levels were monitored in BPAEC at varied confluence. As shown in Fig. 4, glutathione levels decreased as cells reach confluence and the rate of growth declines. Similarly, arresting BPAEC by serum starvation also decreased glutathione levels (data not shown). Thus, growing BPAEC appear to have higher cellular glutathione levels compared with arrested (yet nonapoptotic) cells.

Effects of H_2O_2 on MAP kinases

Extracellular-regulated kinase (ERK) or p44/42 MAP kinase has been shown to regulate cell growth (10). Thus, we studied the effects of various concentrations of H_2O_2 on ERK activity. We found that treatment of BPAEC with high levels of H_2O_2 (1 m*M*, 100 μ*M*, and 30 μ*M*) caused a strong activation of ERK as monitored using a phospho-specific antibody (Fig. 5). ERK protein levels were not different between samples (data not shown). The kinetics of activation by these con-

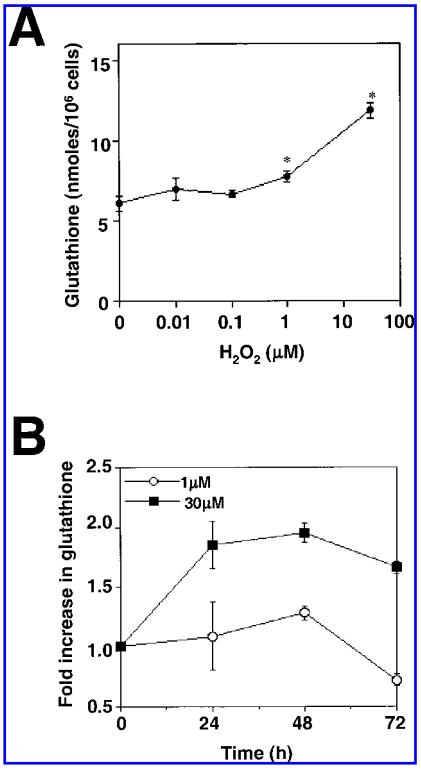

FIG. 3. Effects of H_2O_2 on cellular glutathione content. (**A**) BPAEC were treated with varied concentrations of H_2O_2 for 48 h in medium containing 2% FBS. Levels of total glutathione were determined as described in Materials and Methods. Values represent means ± S.E. *Values different from the 0 μ*M* H_2O_2 value at $p < 0.05$. (**B**) BPAEC were treated with 1 or 30 μ*M* H_2O_2 for the durations indicated. The graph shows means ± SE of fold increase in glutathione.

centrations of H_2O_2 was different as 1 m*M* rapidly activated ERK within 10 min, whereas lower levels required 20 min for activation. Further, ERK activation induced by 100 μ*M* H_2O_2

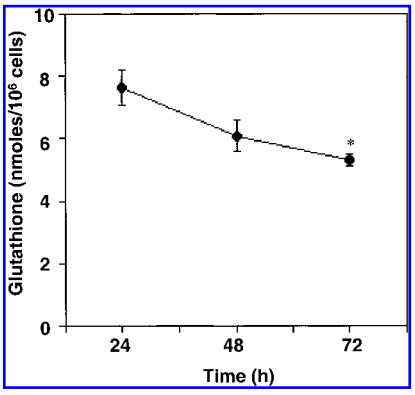

FIG. 4. Effects of cell density on glutathione content. BPAEC were grown for the durations indicated. Levels of total glutathione were determined as described in Materials and Methods. Values represent means ± SE. *Value different from the 24-h value at $p < 0.05$.

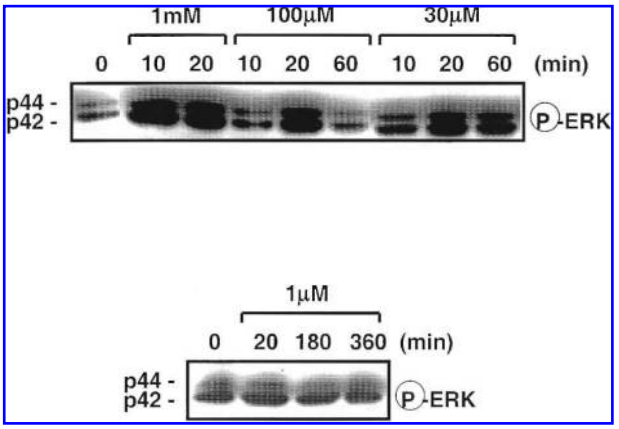

FIG. 5. Effects of H_2O_2 on ERK. BPAEC were treated with varied concentrations of H_2O_2 for the durations indicated. Cell lysates were prepared and the phosphorylation of ERK (p44 and p42) was monitored by western blot using a phospho-specific antibody.

was transient, ceasing by 60 min, whereas that induced by 30 μ*M* H_2O_2 was sustained for 60 min. In contrast, low levels of H_2O_2 (≤1 μ*M*) failed to activate ERK at all time points examined (Fig. 5).

Consistent with these results, an inhibitor of MAP kinase kinase (MEK), U0126 (10 μ*M*), failed to block cell growth induced by 1 μ*M* H_2O_2 (data not shown), suggesting the absence of the MEK-ERK pathway in the mechanism of growth-promoting actions by low levels of H_2O_2 in BPAEC. Similarly, the low levels of H_2O_2 did not activate p38 MAP kinase or c-Jun N-terminal kinase (JNK) (data not shown), excluding the involvement of these signaling pathways.

To determine further the mechanism of increased cellular glutathione content by H_2O_2 especially at low levels, we studied the effects of various doses of H_2O_2 on transcription factor activation. We have previously shown that bleomycin-induced increase in glutathione is through the up-regulation of γ-glutamylcysteinyl synthetase that is regulated by NF-κB and factors that bind to ARE (11). H_2O_2 (1–100 μ*M*), however, did not activate NF-κB DNA-binding activity in BPAEC (data not shown). The DNA-binding activity toward ARE, on the other hand, was activated by 1 and 30 μ*M* H_2O_2 as monitored by EMSA (Fig. 6). H_2O_2 at 30 μ*M* transiently activated the ARE binding with a peak at 2 h, whereas the activation by 1 μ*M* H_2O_2 was slower and did not occur until 3 h.

DISCUSSION

The major finding of the present study is that oxidative stress increases the levels of total glutathione in nontransformed, nonimmortalized culture of BPAEC at low passages. Although varied concentrations of H_2O_2 can exhibit biphasic actions on cell growth, where a low concentration (1 μ*M*) induces proliferation and higher concentrations (>10 μ*M*) promote cell death, the levels of cellular glutathione were increased at both growth-promoting and cell death-inducing concentrations of H_2O_2. High and low concentrations of H_2O_2

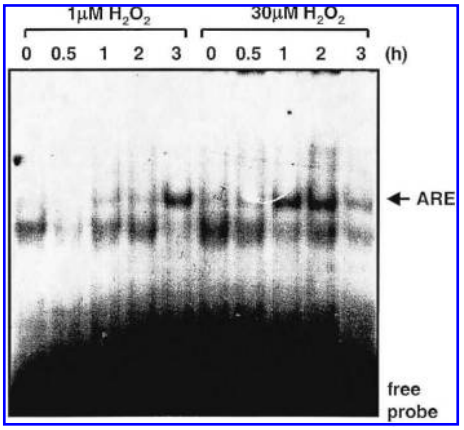

FIG. 6. Effects of H_2O_2 on ARE binding. BPAEC were treated with H_2O_2 for the durations indicated. Nuclear extracts were prepared, and DNA-binding activity toward the ARE sequence was monitored by EMSA.

activated ARE-binding factor(s), which may participate in the regulation of glutathione synthesis.

Reduced cellular glutathione levels have previously been shown to be associated with growth arrest by TGFβ (17), and the present study demonstrates that the levels of glutathione decrease as cell density increases. Both TGFβ and increased cell density have been shown to produce H_2O_2; however, the levels of H_2O_2 produced in these situations are even smaller than exogenously added 1 μ*M* H_2O_2. Nevertheless, these findings show that, in low-passage BPAEC, increased glutathione can be associated with either cell proliferation or cell death, whereas decreased glutathione is associated with cell growth arrest.

Our findings that cell growth- and death-inducing concentrations of H_2O_2 increased glutathione is in contrast to the previous report that H_2O_2-induced cell growth and death were associated with decreased cellular glutathione in various transformed cell lines, such as BHK-21 baby hamster kidney fibroblasts (5). In the lung where cells are often subjected to oxidative stress, glutathione levels may be enhanced as a defense mechanism. Interestingly, although Burdon and coworkers found decreased levels of cellular glutathione in response to high and low doses of H_2O_2, they also found that both doses induced lipid peroxidation products in the BHK-21 cell line (6), indicating that in both cases oxidative damage was produced. Our findings of increased total cellular glutathione in response to either high or low concentrations of H_2O_2 in the low-passage BPAEC may be the result of different cellular responses to oxidative stress of primary lung cells compared with a cell line from other tissues.

Although future work is needed to determine the exact mechanisms of the increase in glutathione by high levels of H_2O_2, our findings suggest that ERK and factors that bind to ARE may be involved, perhaps to up-regulate the gene transcription of γ-glutamylcysteinyl synthetase, a key enzyme that synthesizes glutathione (12). We have previously found that bleomycin increases the levels of glutathione in BPAEC via the up-regulation of γ-glutamylcysteinyl synthetase through mechanisms involving ERK and ARE-binding proteins (11).

The mechanism of the increase in glutathione by growth-promoting, low concentrations of H_2O_2 may also involve ARE-binding factors. We examined the effects of 1 μ*M* H_2O_2 on various signal transduction mechanisms, including ERK, p38 MAP kinase, JNK, and NF-κB, but none of these factors was activated. Interestingly, we found that 1 μ*M* H_2O_2 activated ARE DNA-binding activity. Our previous study has shown that the ARE binding complex in BPAEC consists of Nrf-1, Nrf-2, and at least one unidentified protein, possibly a small maf (11). Thus, H_2O_2 may activate one or more of these proteins in a MAP kinase-independent manner, which could positively regulate enzymes such as γ-glutamylcysteinyl synthetase, resulting in increased cellular glutathione.

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ABBREVIATIONS

ARE, antioxidant response element; BPAEC, bovine pulmonary artery endothelial cells; EMSA, electrophoretic mobility shift assay; ERK, extracellular-regulated kinase; FBS, fetal bovine serum; H_2O_2, hydrogen peroxide; JNK, c-Jun N-terminal kinase; MAP, mitogen-activated protein; MEK, mitogen-activated protein kinase kinase; NF-κB, nuclear factor-κB; RPMEC, rat pulmonary microvascular endothelial cells; TGFβ, transforming growth factor-β1. TNB, 5-thio-2-nitrobenzoate.

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