Altered Regulation of G₁ Cyclins in Oxidant-induced Growth Arrest of Lung Alveolar Epithelial Cells

ACCUMULATION OF INACTIVE CYCLIN E-CDK2 COMPLEXES

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The alveolar surface of the lung is a major target for oxidant injury, and its repair following injury is dependent on the ability of its stem cells, the type 2 cells, to initiate proliferation. From previous studies it is likely that events located before the entry into the S phase of the cell cycle and involving several components of the insulin-like growth factor system as well as transforming growth factor-β (TGF-β) play a key role in growth regulation of oxidant-exposed type 2 epithelial cells. To gain further insights into these mechanisms, we explored the effects of O₂ exposure on G₁ cyclins and their cyclin-dependent kinases (CDKs). We documented an increased expression of these genes in O₂-treated type 2 cells. However, despite this induction, a dramatic decrease in cyclin E-CDK2 activity, but not in cyclin D-CDK4 activity, was found. The concomitant induction of CDK inhibitory proteins (CKIs), mainly p21[^CIP1], suggests that accumulation of inactive cyclin E-CDK2 activity is due to CKI binding. We also provided evidence that the mechanisms regulating this process involved TGF-β as anti-TGF-β antibody treatment was able to reduce the oxidant-induced inhibition of cyclin E-CDK2 activity. Taken together, these results suggest that oxidants may block entry into S phase by acting on a subset of late G₁ events whose alterations are sufficient to impaire the activation of cyclin E-CDK2 complexes.

Oxidants are involved in a large variety of pulmonary diseases. This is well explained by the location and anatomy of the lung which provides an extensive surface area available to react with all sources of reactive O₂ species (1, 2). Among the various cell types which compose the lung, the epithelial cells of the alveolar structure appear to be a major target for oxidant injury (3–6). These epithelial cells are of two types: type 1 and type 2 cells. It is now well established that type 2 cells are the stem cells of the alveolar epithelium, and that repair of damaged alveolar surface is dependent on their ability to replicate and to provide additional cells that have the potential to undergo transition into type 1 cells. After oxidant injury, the rapidity of initiation of type 2 cell proliferation is crucial for a proper healing, as delay in the reepithelialization process has been implicated in the development of pulmonary fibrosis (7–9). Therefore, characterization of the mechanisms involved in the block of type 2 cell replication by oxidants and in its reversibility appears to be critical for the understanding and management of many lung diseases associated with oxidative stress.

Cell proliferation is a highly regulated process involving a series of molecular events which follow an ordered sequence ensuring that, before division, cells have completed DNA replication and chromosome segregation. Cell cycle progression involves major checkpoints located before the entry into S phase and into M phase (10–14). When cells are exposed to DNA damaging agents such as oxidants, a feedback control is activated that acts as brake on cell cycle to inhibit progress through the G₁-S transition until DNA repair is completed (15–17).

The decisions of cells to enter S phase or to arrest in G₁ is determined by a complex integration of proliferative and anti-proliferative signals which involve extracellular factors and intracellular molecules, and which converge to key regulators of the cell cycle machinery: the cyclin-dependent kinases (CDKs) (18). Active CDKs are composed of a catalytic subunit and a regulatory subunit termed cyclin. The G₁ cyclins include cyclins D (D₁, D₂, and D₃) and cyclin E (19, 20). The D-type cyclins activate mostly CDK4, and cyclin E forms complex with CDK2. The involvement of these various cyclins during G₁ progression is not completely defined, but it is likely that the D-type cyclins may act earlier in G₁ than cyclin E. Regulation of G₁ cyclin CDK activity is also dependent on CDK inhibitory proteins (CKIs) which can bind and inactivate cyclin-CDK complexes (21–23). Several inhibitory proteins have been identified. They include p21 (a protein of M₈, 21,000 also named pClP1, WAF1, or Sdi1) and p27 (also named KIP1). CKIs have been reported to be induced upon DNA damage and to mediate G₁ cell-cycle arrest; they can function as tumor suppressor proteins, most likely by inhibiting the ability of cyclin-CDKs to phosphorylate retinoblastoma protein (pRb) (24, 25). Moreover, the transforming growth factor-βs (TGF-βs), a group of proteins that mediate inhibition of cell proliferation by inducing growth arrest in G₁, have been shown to regulate the activities of several CKIs (26, 27).

In recent studies, we have documented the involvement of...
several components of the insulin-like growth factor (IGF) system, mainly the IGF-binding protein 2 (IGFBP2), IGF II and the type 2 IGF receptor, as well as of TGF-β in the inhibition of DNA synthesis by type 2 alveolar epithelial cells exposed to O₂ (28).

Interestingly, these factors have been shown in various cell systems to act in late G₁, and from several reports it is likely that events operating at the end of G₁ and/or during the G₁/S transition play an important role in type 2 cell regulation of proliferation. To gain some insights into the mechanisms involved in the block of type 2 cell replication by oxidants and in its reversibility, we explored the effects of O₂ exposure on G₁ cyclins and their CDKs. In the present work, we report an increased expression of the various G₁ cyclins and CDKs in O₂-treated cells. Despite this induction, a dramatic decrease in cyclin E-CDK2 activity was observed in O₂-exposed cells. The concomitant induction in the CKGs, p21<sup>CD24</sup> and p27<sup>KIP1</sup>, by oxidant exposure suggests that accumulation of inactive cyclin E-CDK2 complex is due to CKI binding. The previous findings that oxidants induce TGF-β expression, that TGF-β produces G₁ arrest in a rat type 2 and in human lung fibroblasts, and that the present observation that anti-TGF-β antibodies reduce the oxidant-induced inhibition of cyclin E-CDK2 activity provide evidence that TGF-β may be involved in this process. All these results suggest that oxidants may block entry into S phase by acting on a subset of late G₁ events whose alterations are sufficient to impair the activation of cyclin E-CDK2 complex.

MATERIALS AND METHODS

Cyclins in Lung Epithelial Cells Exposed to Oxidants

For protein electrophoresis and immunoblotting, cellular proteins were analyzed as described previously (33). Duplicate dishes were used for each experimental condition. One dish was used for cell number determination. The cells from the other dish were washed with cold phosphate-buffered saline and scraped in 2× Laemmli sample buffer, the volume of buffer being adjusted to cell number. Equal volumes of samples were loaded for each experimental condition, and proteins were separated by SDS-PAGE (10% acrylamide). Western blot experiments were performed by transferring the proteins onto 0.45-μm nitrocellulose membranes (Bio-Rad) for 1 h at 130 V. Immunoblotting was performed by first saturating the nitrocellulose sheet for 4 h at room temperature in TBS (20 mM Tris-HCl, pH 7.6, 137 mM NaCl) containing 0.2% (TBS-T) and 100 mM (TBS-T) EDTA, 1 mM dithiothreitol. The membranes were then washed three times with TBS-T buffer and incubated for 1 h at 37°C with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Amersham Corp.), diluted 1:1000 in milk-TBS. The membranes were then washed three times in TBS-T buffer, after which they were incubated for 2 min at room temperature in chemiluminescence reaction detection reagents (ECL Western blotting, Amersham Corp.). The membranes were then exposed to autoradiography film (Hyperfilm-ECL, Amersham Corp.).

Immunoprecipitation and Kinase Assays—Cells (800×10<sup>3</sup>) were washed three times with phosphate-buffered saline and lysed by addition of 40 μl of lysis buffer (250 mM NaCl, 50 mM HEPES, pH 7.0, 5 mM EDTA, 1 mM dithiothreitol, 0.1% Nonidet P-40, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 50 μg/ml phenylmethylsulfonyl fluoride, 2 mM sodium pyrophosphate, 1 mM sodium orthovanadate). The lysates were clarified by centrifugation at 10,000×g for 10 min at 4°C and incubated at 4°C overnight with either anti-cyclin E antibody or anti-CDK4 antibody. Cyclin-CDK complexes were then isolated by incubation at 4°C for 1 h with 50 μl of either protein A-Sepharose beads (Pharmacia Biotech Inc.) or GSH-agarose beads (Pharmacia). The beads were then washed and incubated for 30 min at 30°C in 25 μl of reaction buffer (50 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 1 mM dithiothreitol) in the presence of either 5 μg of histone H1 (Boehringer Mannheim) or 1 μg of GST-pRb substrate (a gift from Dr. Ewen, Dana Farber Institute, Boston, MA), 1 μCi of [γ-<sup>32</sup>P]ATP (4500 Ci/m mole) and 50 μl ATP (24, 34, 35). Reactions were stopped by adding 40 μl of 2× SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.025% bromophenol blue, 5% β-mercaptoethanol). The samples were then boiled for 5 min before loading onto SDS-PAGE. Labeled proteins were detected by autoradiography and quantified with densitometric scanner.

Statistical Methods—Results are reported as mean ± S.E. Data were analyzed using analysis of variance followed, when adapted, by a Bonferroni t test for multiple comparisons against control conditions. Significance is assigned for p < 0.05.
**RESULTS**

**Effects of Hyperoxia on G₁ Cyclins CDK2 and CDK4 mRNA Expression**—In previous studies, we have reported that exposure of type 2 cells to hyperoxia led to a rapid and reversible inhibition of cell proliferation (30). No increase in cell number was observed after 24 h in 95% O₂, and this was associated with a rapid decrease in the percent of labeled nuclei. When cells were replaced under normoxia after a 48-h O₂ exposure, they rapidly resumed proliferation within 24–48 h with a labeling index of more than 90% after 48 h under control conditions. Using the same experimental conditions, we first investigated whether O₂-induced block of proliferation was associated with changes in G₁ cyclins and/or CDKs (CDK2 and CDK4) expression at the level of mRNA. For these experiments, RNA from exponentially growing cells cultured under standard conditions, from cells exposed to hyperoxia (95% O₂) for 24 or 48 h, and from cells cultured for 48 h under hyperoxia and then under normoxia for an additional 24 h period, was extracted and studied by Northern blotting. As shown in Figs. 1 and 2, the levels of the studied mRNA increased upon O₂ exposure. A maximal effect was observed after 48 h under hyperoxia with a 3–8-fold increase in G₁ cyclins (cyclin D1, D2, D3, and E) mRNA, as well as a 8–9-fold increase in CDK2 and CDK4 mRNA. When O₂-exposed cells were returned to normoxia, a rapid decrease in the mRNA levels was found. It is important to point out that in other studies performed using the same experimental protocol, absence of modifications as well as decrease in mRNA levels for various genes was observed in O₂-treated cells (28, 30). This indicates that the increase in G₁ cyclins and CDK2 and CDK4 expression documented in the present study is not a common response to oxidants.

**Effects of Hyperoxia on G₁ Cyclins CDK2 and CDK4 Protein**—To determine whether O₂-induced G₁ cyclins and CDK2 and CDK4 mRNA were associated with an increase in the corresponding proteins, protein extraction was performed from proliferative cells, O₂-exposed cells, and O₂-exposed cells for 48 h and then cultured under normoxia for 24 or 48 h. Western analysis are shown in Figs. 3 and 4. A significant increase in protein levels upon O₂ exposure was observed for cyclin E, with a maximal effect after 48 h. This effect was reversible; when O₂-treated cells were allowed to resume proliferation after return to control conditions, a rapid decrease in cyclin E protein level was observed. For cyclin D1, D3, CDK2, and CDK4, minor changes were observed upon O₂ exposure and in cells returned to normoxia after a 48-h culture under hyperoxia, but they did not reach statistical significance. As mentioned above, increase in G₁ cyclin and their CDK at the level of protein cannot be considered as a common response to oxidants, as dramatic decrease in the levels of several newly synthesized proteins such as histone proteins and thymidine kinase was documented in previous studies (30). Finally, using our experimental protocols, no cyclin D2 protein could be detected.

**Effects of Hyperoxia on Cyclin-CDK Kinase Activities**—In various cell systems, the levels of G₁ cyclins and their CDKs appear to be modulated during cell cycle progression. Increased expression is observed during the G₁ phase and the transition to S phase and is associated with proliferation. To determine whether induction of G₁ cyclins and their CDKs in O₂-treated cells was associated with changes in protein kinase activity of different cyclin-CDK complexes, the cyclin E-CDK2 complex was assayed for its kinase activity after immunoprecipitation against cyclin E by use of histone H1 as the substrate. Results...
are shown in Fig. 5. When compared to the activity observed in proliferating cells, O₂ treatment led to a rapid decrease in histone H1 kinase activity, which was almost completely abolished after 48 h of hyperoxia. When O₂-treated cells were allowed to resume proliferation in normoxia, histone H1 kinase activity returned rapidly to the level observed in exponentially growing cells. The cyclin D-CDK4 complex was assayed for its kinase activity toward pRb. When GST-pRb was used as a substrate, immunoprecipitations obtained with antibodies to CDK4 from extracts of cells cultured under normoxia or hyperoxia did not exhibit consistent modifications. As shown in Fig. 6, lysates from cells exposed to O₂ generated a level of kinase activity which was not different from cells cultured under control conditions or from cells allowed to resume proliferation after O₂ exposure.

Enhanced Expression of CKIs in O₂-induced Growth Arrest of Type 2 Cells—To examine whether CKIs could be involved in the down-regulation of G₁ cyclin-CDK complex activity induced by O₂, proliferating cells, cells treated with O₂, and cells returned to normoxia after 48 h hyperoxia were assayed for p21<sup>WAF1</sup> and p27<sup>KIP1</sup> mRNA expression by Northern blot analysis (Fig. 7). A dramatic increase of both p21<sup>WAF1</sup> and p27<sup>KIP1</sup> mRNA was observed in O₂-exposed cells, with a maximal effect after 48 h. Furthermore, this effect was reversible with decreased mRNA in cells allowed to resume proliferation after O₂ treatment. To determine whether increased mRNA expression was associated with an increase in protein levels, cells under the same experimental conditions were assayed for p21<sup>WAF1</sup> and p27<sup>KIP1</sup> proteins by Western blot analysis. As shown in Fig. 7, a significant induction of proteins was observed only for p21<sup>WAF1</sup>. Indeed, the increase in p27<sup>KIP1</sup> protein levels in O₂-treated cells observed on the autoradiogram of Fig. 7 did not reach statistical significance when the results of three independent experiments were analyzed.

Involvement of TGF-β in O₂-induced Inhibition of Cyclin E-CDK2 Complex Activity—To gain some insight into the mechanisms of cyclin E-CDK2 complex inactivation in type 2 cells growth-arrested by hyperoxia exposure, we asked whether TGF-β could be involved in this process. This question was based on the fact that TGF-β has been shown to be a potent inhibitor of type 2 cell proliferation, that it was induced by O₂ exposure in these cells, and that it regulates G₁ cyclin-CDK complex activity at multiple levels, including the induction of CKIs (26, 28, 36).

Based on the reported actions of TGF-β on the regulation of either G₁ cyclins, their CDKs or the CKIs, we focused on the effect of TGF-β on cyclin E-CDK2 complex activities. For these experiments, type 2 cells were exposed to 95% O₂ in regular medium, in medium with anti-TGF-β1 antibody at a concentration defined in previous studies, or in medium containing normal chicken IgG. After 48 h of hyperoxia, cells were washed and prepared for cyclin kinase activity using histone H1 as the substrate. Results are shown in Fig. 8. A dramatic decrease in histone H1 activity of the cyclin E complex was observed in
cells cultured in regular medium. As determined by densitometric analysis, hyperoxia exposure of cells in the presence of 100 μg/ml anti-TGF-β1 antibody resulted in a 2-4-fold increase in kinase activity when compared with cells exposed to O₂ without antibody or in the presence of normal chicken IgG.

Interestingly, we confirmed that in our system TGF-β1 was able to induce p21CIP1 (data not shown). In previous studies, we have documented a strong induction of TGF-β1 expression in cells growth-arrested by hyperoxia exposure (95% for 48 h). It is now well documented that TGF-β1 interacts with a set of cell surface receptors, including the type I and type II receptors, which are essential for signal transduction. To determine whether O₂ exposure was also associated with increased expression of these receptors, RNA was extracted from type 2 cells cultured under normoxic conditions, in hyperoxia (24 and 48 h), and from cells returned to normoxia after O₂ exposure. Results are shown in Fig. 9; block of proliferation induced by O₂ was associated with a dramatic induction of both type I and type II TGF-β receptors (37). This effect of O₂ was reversible as shown in the experimental conditions where cells were allowed to resume proliferation after hyperoxia. Again, as discussed above, from results obtained in studies of unrelated receptors, the changes in type I and type II TGF-β receptor expression documented in O₂-treated cells cannot be considered as a common response to oxidants (28).

FIG. 4. Effects of hyperoxia on CDK2 and CDK4 protein levels. Cellular proteins were extracted from exponentially proliferating cells cultured under air (1), under hyperoxia (95% O₂) for 24 h (2) or 48 h (3), or under hypoxia for 48 h and then under normoxia for 24 h (4) or 48 h (5). Proteins were analyzed by immunoblotting and were fractionated on SDS-PAGE, transferred to nitrocellulose, and probed with the corresponding antibody, as described under "Materials and Methods." A, autoradiogram of signals for CDK2 (33 kDa) and CDK4 (33 kDa) proteins. B, the histogram shows a quantitative representation of CDK2 and CDK4 protein levels obtained from laser densitometric analysis of three independent experiments. Results were expressed in arbitrary densitometric units.

FIG. 5. Effects of O₂ on histone H1 kinase activities of cyclin E-CDK2. Cell lysates were prepared from exponentially proliferating cells cultured under air (1), under hyperoxia (95% O₂) for 24 h (2) or 48 h (3), or under hypoxia for 48 h and then under normoxia for 24 h (4) or 48 h (5). Cyclin E-associated kinase activity was determined by using histone H1 as a substrate as described under "Materials and Methods." The reaction products were electrophoretically separated on denaturing gels, and phosphorylated proteins were detected by autoradiography (A). B, the histogram shows a quantitative representation of phosphorylated histone H1 levels obtained from laser densitometric analysis of three independent experiments, with results expressed in arbitrary densitometric units. *, p < 0.05 versus control conditions.

FIG. 6. Effects of O₂ on the pRb kinase activities of cyclin D-CDK4. Cell lysates were prepared from exponentially proliferating cells cultured under air (1), under hyperoxia (95% O₂) for 24 h (2) or 48 h (3), or under hypoxia for 48 h and then under normoxia for 24 h (4) or 48 h (5). CDK4-associated kinase activity was determined by using purified bacterial GST-pRb fusion protein as a substrate, as described under "Materials and Methods." A, the reaction products were electrophoretically separated on denaturing gels, and phosphorylated proteins were detected by autoradiography. B, the histogram shows a quantitative representation of phosphorylated pRb levels obtained from laser densitometric analysis of the upper bands (the lower molecular weight bands are nonspecific bands and represent contaminating species).
Emphasis on oxidative stress and its effects on cell cycle machinery in oxidant-induced response. We have shown that inhibition of lung alveolar epithelial cell proliferation by hyperoxia alters expression of the late G1 cyclin CDKs, as cell growth arrest is associated with decreased cyclin E-associated kinase activity even though cyclin E-CDK2 complexes were abundant. Furthermore, study of the mechanisms involved in this reduced activity strongly suggests that it may be due to oxidant-induced increase in the CKI p21<sup>CIP1</sup>, which may in turn be secondary to induction of TGF-β.

**DISCUSSION**

Oxidant injury represents a major cause of lung diseases, and understanding of the mechanisms associated with the repair process is crucial for characterization of the key factors involved in the proper remodeling of the respiratory structures. In the present work, we focused on the epithelial cells of the pulmonary alveolus, a site of maximum oxidant exposure in disease. Using a model system of reversible oxidative stress, we have documented involvement of several components of the cell cycle machinery in oxidant-induced response. We have shown that inhibition of lung alveolar epithelial cell proliferation by hyperoxia alters expression of the late G1 cyclin CDKs, as cell growth arrest is associated with decreased cyclin E-associated kinase activity even though cyclin E-CDK2 complexes were abundant. Furthermore, study of the mechanisms involved in this reduced activity strongly suggests that it may be due to oxidant-induced increase in the CKI p21<sup>CIP1</sup>, which may in turn be secondary to induction of TGF-β.

Exposure to oxidants causes intracellular production of reactive O₂ species which interact with and modify a spectrum of biomolecules including DNA. DNA damage is thought to be a critical event in governing responses of cells to oxidants. Indeed, in most cells one effect of DNA damage is a transient inhibition of DNA synthesis to allow DNA repair. Therefore, specific responses of mammalian cells to oxidative stress include activation of DNA repair enzymes, as well as alterations in the expression of a series of genes involved in the cell cycle. These responses lead to a G₁-S cell cycle arrest which provides cells with increased time to restore DNA damage before DNA replication (11, 12, 38). Among the positive regulators of cell cycle progression from G₁ to S are the G₁ cyclins. From recent studies, distinct regulation and roles of the various G₁ cyclins, D-type cyclins, and cyclin E are proposed. Synthesis of D-type cyclins appears to be rapidly induced by growth factor stimulation and exhibits moderate changes throughout the cell cycle. In contrast, induction of cyclin E is observed later in G₁, with expression maximal at the G₁-S transition (18–20). Progression through G₁ is also associated with distinct patterns of activation of the complexes the G₁ cyclins form with members of the CDK family. Activities of D-type cyclin CDKs are first detected in mid-G₁ phase and increase as cells approach the G₁/S boundary. Later in G₁, cyclin E forms complex with CDK2 whose activity is maximally induced just before entry into the S phase. D-type cyclins and cyclin E appear to perform different functions in promoting onset of DNA replication, with D-type cyclins acting prior to cyclin E (39–41).

In the present work, study of G₁ cyclin and CDKs expression showed that oxidant exposure was associated with increased levels of the various mRNAs. However, analysis of the corresponding proteins indicated that a significant increase was observed only for cyclin E. Interestingly, despite accumulation of cyclin E protein, cyclin E-associated kinase activity appeared to be deficient in O₂-treated cells. These data raise two issues. First, oxidants can alter G₁ cyclin and CDK gene expression by acting at various levels: transcriptional and post-transcriptional. Modulation of gene expression by transcriptional mechanisms in situations of oxidative stress has been reported in
several studies. Indeed, several transcription factors have been shown to respond to the intracellular concentrations of reactive O₂ species (42). They include AP-1 and NF-κB. AP-1 can be activated by various stimuli known to alter the redox state of the cells. Active AP-1 complexes may, then, bind DNA target sequences in many promoters, including promoters of antioxidative enzymes (43, 44). The transcription factor NF-κB responds directly to oxidative stress, and, by binding to its cognate DNA sequence, can activate expression of a wide range of genes, including cytokine and growth factor genes (45). In addition, oxidants can control gene expression at the post-transcriptional level by inducing modifications of preexisting proteins, which may be involved in translation machinery by binding to specific regulatory elements of mRNAs (46, 47). These modifications include poly(ADP-ribose)ylation of specific proteins, as poly(ADP-ribose)polymerase has been shown to be activated upon oxidant-induced DNA damage and to act on a large variety of proteins (48, 49).

The second issue relates to the main G₁ checkpoint controlled by oxidants which may be located just before the onset of DNA synthesis. This is suggested by the results presented herein showing that synthesis of either G₁ cyclins or their CDKs was not decreased by oxidants, and that in O₂-treated cells cyclin E-CDK complex activity was altered but that activation of cyclin D-CDK4 would allow the activation of cyclin E-CDK2 complex. Moreover, they provided evidence that the protein responsible for the activity capable of inactivating cyclin E-CDK2 complex was p21^{CIP1}. Induction of p21^{CIP1} protein by irradiation was also documented in other cell lines (52, 53). Additional data support the hypothesis that p21^{CIP1} may be predominantly involved in the failure to activate cyclin E-CDK2 in O₂-treated cells. First, as reported above, significant accumulation of the corresponding protein was only documented for p21^{CIP1} despite increased expression of both p21^{CIP1} and p27^{KIP1}. Second, it is now believed that p27^{KIP1} interacts mostly with D-type cyclins and CDK4 and more weakly with cyclin E and CDK2, and that binding of p27^{KIP1} to cyclin D-CDK4 would allow the activation of cyclin E-CDK2 complexes at the G₁-S transition (54–56). Lastly, in situations of cell cycle block induced by DNA-damaging agents, a role of p21^{CIP1} in inactivation of cyclin E-CDK2 complex has been predominantly documented (51–53).

Reports from several laboratories have emphasized a role of p53 protein in the G₁-S cell cycle arrest in cells exposed to DNA-damaging agents (38). Indeed, in wild type cells, irradiation increased the levels of p53 and induced G₁ arrest; this arrest was not observed in cells lacking p53. It is now well documented that p53 coordinates multiple responses to DNA damage. It functions as a transcription factor and can stimulate DNA repair through stimulation of the GADD45 gene expression; its action on G₁-S arrest involved transcriptional activation of p21^{CIP1} gene (57, 58). On the other hand, if expression of p21^{CIP1} has been reported to be regulated by p53,
recent studies have also documented p53-independent pathways of p21cip1 induction, which can occur at both the transcriptional and posttranscriptional levels (59, 60). A possible involvement of p53 in the present system remains to be determined (61).

The last conclusion that arises from the results presented in this study relates to the involvement of TGF-β in the inactivation of cyclin E-CDK2 complexes upon oxidant exposure. The hypothesis of a role of TGF-β in this process was based on several reported works. First, TGF-β is a potent growth inhibitor of epithelial cells, and, in previous study, we have provided evidence that this factor was strongly induced in cells growth-arrested by O2 treatment (28). Furthermore, in the present work, we have been able to document an increased expression under hyperoxia of the two types of TGF-β receptors, the type I and the type II, which are known to be essential for signal transduction. Second, TGF-β stops cell proliferation by arresting progression through the late portion of G1 (62). It is now proposed that growth arrest by TGF-β might be mediated by the G1 cyclins, the CDKs, and alteration in retinoblastoma protein phosphorylation. In various epithelial cell lines, it has been shown that TGF-β modulates G1 cyclins and associated kinase activities at multiple levels. Using human keratinocytes, Geng and Weinberg (36) showed that TGF-β inhibited expression of cyclin E, CDK2, and CDK4. In another study, Koff et al. (26) reported that TGF-β could block the formation of active cyclin E-CDK2 complexes in cells that already expressed cyclin E. From these studies, it has been proposed that TGF-β can stop cell cycle progression by either inhibiting expression of G1 cyclins and their CDKs, or preventing the formation of active cyclin-CDK complexes. In the present work, the results of experiments with anti-TGF-β antibody strongly suggest that TGF-β may be involved in the inactivation of cyclin E-CDK2 complexes in cells growth-arrested by oxidant exposure. TGF-β could alter cyclin E-CDK2 activities via its effect on p21cip1. This is first supported by our results showing that O2 treatment induced p21cip1 expression, at both mRNA and protein levels. Also, in a recent study, Li et al. (63) have reported that TGF-β may be increased to p21cip1 levels in colon cancer cell lines, this effect was also found in our system. These authors also showed that p21cip1 induction correlated with reduced cyclin E-associated kinase activity, and that p21cip1 was physically associated with cyclin E in these cell lines.

Results presented in this study indicate that proliferation arrest of lung alveolar epithelial cells by oxidants is associated with an inhibition of cyclin E-CDK2 complex activities, which prevents entry into S phase and DNA replication. Mechanisms regulating this process likely involve TGF-β with the possible following sequence of events. Induction of TGF-β was found to be a rapid response of cells when exposed to hyperoxia, and may represent the first event in a cascade (28). Subsequently, TGF-β, through interactions with its cell surface receptors which were also dramatized induced by oxidants, would trigger increased p21cip1. p21cip1 would then prevent activation of cyclin E-CDK2 complex, and this inactivation ensures a pause in cell cycle progression allowing the cell to repair any DNA damage induced by oxidants before proceeding. Moreover, in the results presented herein and together with a recent report in the literature suggesting that cyclin E positively regulated p53 transcription activation, it is also possible to include p53 in this hypothetical scheme (64). Indeed, in O2-treated cells, an increased protein production was found for cyclin E but not for the other G1 cyclins. The reason for cyclin E protein accumulation could be to allow its combination with the CDK2 available in the cells. The cyclin E-CDK2 complex could then activate p53, which in turn could initiate the complex by transactivation of p21cip1 and activate DNA repair genes. This sequence of events with initial activation of TGF-β may represent a rapid protective mechanism for cells in situations of oxidative stress. The potential importance of TGF-β involvement in this process is further supported by our recent data indicating the existence of a regulatory link between components of the IGF system (mainly IGFBP-2) and TGF-β in hyperoxic control of cell proliferation (28) Future studies need now to directly explore this hypothesis in order to understand the role and the mechanisms of action of TGF-β in the growth arrest of epithelial cells exposed of oxidative stress.
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