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Role for NF-κB in mediating the effects of hyperoxia on IGF-binding protein 2 promoter activity in lung alveolar epithelial cells

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Abstract

The surface of the pulmonary alveolus is a major target for oxidant injury, and its proper repair following injury is dependent on the proliferative response of the stem cells of the alveolar epithelium, the type 2 cells. In previous studies on the mechanisms controlling this response, we have documented involvement of several components of the IGF system, and mainly of the IGF binding protein-2 (IGFBP-2). We have provided evidence that this binding protein was associated with inhibition of DNA synthesis of type 2 cells exposed to oxidants and that its expression was regulated mostly at the level of transcription. In the present study, we focused on the factors involved in this regulation. From examination of the IGFBP-2 gene promoter sequence which revealed the presence of four potential binding sites for transcription factors of the NF-κB/Rel family, we hypothesized that NF-κB might be involved in the transcriptional activation of IGFBP-2 in oxidant-exposed cells. Data reported herein demonstrated that NF-κB activated IGFBP-2 promoter in transient transfection assays, and that exposure of cells to hyperoxia was associated with accumulation of the active form of NF-κB. Using gel shift analysis, we documented in O_2-treated cells an increased binding to the four NF-κB binding sites. We also showed that accumulation of NF-κB was associated with a decrease in the inhibitory molecule IκB-α. Based on the current knowledge on NF-κB regulation, it is likely that in a number of situations associated with injury of lung alveolar epithelial cells signaling events involving accumulation of NF-κB converge to activate IGFBP-2 and to block entry into S phase.

1. Introduction

Among the structures of the respiratory system, the surface of the alveolus represents a major target for oxidant injury as it is directly exposed to the various forms of reactive oxygen (O_2) species which can be produced by inflammatory cells, by chemical or pharmacological agents, as well as by environmental particles [1–3]. The epithelium of the alveolar structure of the lung is composed of two types of epithelial cells: type 1 and type 2 cells [4]. Only the type 2 cells have the ability to replicate and they are considered as the stem cells of this epithelium as they can also undergo transition into type 1 cells [5–7]. Therefore, the process of type 2 cell proliferation is a crucial element for lung homeostasis. Any impairment in type 2 cell replication following oxidant

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injury may lead to an altered reparation of the respiratory epithelium [8,9].

Based on this knowledge, characterization of the mechanisms involved in the block of alveolar epithelial type 2 cell proliferation and in its reversibility appears critical for the understanding and management of many lung diseases associated with oxidative stress. From several reports in the literature, it is likely that events operating late in the G_1 phase of the cell cycle and/or during the G_1/S transition play a key role in type 2 cell regulation of proliferation [10–12]. These events are certainly of importance in type 2 cells exposed to oxidants, as arrest before entry into S phase represents an adequate response of all cells when exposed to DNA damaging agents. This response functions as a checkpoint control and is associated with cell-cycle delays which provide opportunities for DNA repair before replication in order to avoid irreparable mutations [13].

Focusing on the events controlling the entry into S phase of type 2 alveolar epithelial cells, we have documented in previous studies the involvement of several components of the insulin-like growth factor (IGF) system, and mainly of the IGF binding protein-2 (IGFBP-2) [14–16]. We have provided evidence that this binding protein was associated with inhibition of DNA synthesis in cells exposed to oxidants and that its expression was regulated mostly at the level of transcription. Examination of the IGFBP-2 gene promoter revealed the presence of several potential binding sites for transcription factors of the NF-κB/Rel family.

NF-κB is a family of related peptides which can be divided into two groups based on differences in structures and functions [17]. The first group includes p50 and the second p65. Members of both groups can form multiple homo- or heterodimer complexes such as p50-p65 heterodimers. NF-κB is an inducible eukaryotic transcription factor which exists in an inactive form in the cytoplasm of most cells where it is bound to inhibitory proteins IκB [18,19]. NF-κB can be activated in response to a number of factors including viruses, bacteria, oxidants, and inflammatory cytokines [3,20]. These factors trigger the release from IκB allowing migration of the heterodimer to the nucleus where NF-κB binds to DNA and regulates transcription of specific genes [21]. The various pathways leading to NF-κB activation implicate IκB phosphorylation and degradation. At least two different inhibitors seem to be involved: IκB-α and IκB-β [22]. Distinct modes of regulation as well as distinct responses to various NF-κB inducers have been recently reported for these proteins. So far, degradation of IκB-α has been observed in all situations of NF-κB activation. This was not found for IκB-β [23]. IκB functions by inhibiting the DNA binding and the nuclear uptake of associated DNA-binding proteins. The mechanism of this inhibitory action seems to be a direct binding of IκB to the nuclear location signal of p65 [18].

A number of studies have shown that nuclear retention and DNA binding of NF-κB are increased following exposure to a large variety of components which lead to an increased oxidant production. Also recent works have suggested that NF-κB could be involved in growth control [17,24]. Together with the finding that NF-κB binding sites were present in IGFBP-2 promoter, these data prompted us to ask whether NF-κB could play a role in the transcriptional activation of IGFBP-2 in lung alveolar epithelial cells growth-arrested by oxidant exposure. In the present study, we provide data indicating that NF-κB is able to activate the IGFBP-2 promoter. We also document in O_2-exposed cells an accumulation of the active form of NF-κB as well as an increased binding to the four NF-κB binding sites present in the IGFBP-2 promoter. Mechanisms involved in accumulation and activation of NF-κB and IGFBP-2 with block of entry into S phase of lung epithelial cells exposed to oxidative stress are discussed.

2. Materials and methods

2.1. Cells, cell culture conditions

The type 2 cell line used in this study was derived from rat lung neonatal type 2 cells by transfection with SV40-T antigen gene and has been extensively characterized [25]. Cells were grown in Earle's MEM (Gibco BRL, Grand Island, NY) supplemented with 4 mM glutamine, 50 U of penicillin/ml, 50 μg of streptomycin/ml, and 10% fetal bovine serum (FBS) (Eurobio, France). For control conditions, cells were cultured in a 5% CO_2/95% air atmosphere at 37°C.

For hyperoxic conditions, they were cultured in 5% CO_2/95% O_2 atmosphere at 37°C. Under these hyperoxic conditions, cells ceased proliferation after 24 h O_2, as previously described [11,14].

For proliferation studies, cells were plated at a density of 2×10^4 cells/35 mm culture dish. Three days later, the medium was changed and the cells were placed either in control atmosphere or in hyperoxia for the indicated times. Cell number was determined by harvesting the cells with trypsin-EDTA and counting them in triplicate using a hemocytometer [26].

For the study of pyrrolidine dithiocarbamate (PDTC) effects, proliferative cells (50% confluence) were incubated in a medium containing 10^{-5} M or 10^{-4} M of PDTC (Sigma, St. Louis, MO) and exposed to 95% O_2 for 12 h.

For each protocol, three or four independent experiments were performed.

2.2. DNA transfection and in vivo transcription assay

For transfection experiments, the following plasmids were used. The IGFBP-2 promoter plasmid p(−1400/+12) IGFBP-2-LUC was constructed as previously indicated [16]: it contains the fragment of the rat IGFBP-2 promoter extending from +12 bp relative to the start of transcription plus 1.4 kb of 5′-flanking sequence, ligated to a firefly luciferase reporter gene into plasmid pBL-LUC. In addition, the fragment of IGFBP-2 promoter, −580 bp to +12 bp, fused to the promoterless firefly luciferase reporter gene (a gift from Dr. Kuto, Basel, Switzerland) was also used [27]. pRSVLUC (a gift from Dr. Schneid, St. Antoine University, Paris, France) contains the Rous sarcoma virus promoter/enhancer fused to the luciferase reporter gene. pBL-LUC is a promoterless plasmid. pSV40β-gal plasmid expresses the *Escherichia coli lacZ* gene under the control of SV40 promoter (a gift from Dr. Schneid), and was used as an internal control to assess the efficiency of transfection. All plasmids were prepared using plasmid extraction kits (Qiagen, Chatsworth, CA).

Plasmids were introduced into cells using the lipofectine method. Adherent cells (60% confluent) were washed twice with MEM and were incubated for 24 h at 37°C with 10 μl lipofectine solution (1 mg/ml) (Gibco BRL) and 3 ml of serum and antibiotic free medium containing 5 μg of luciferase plasmid and 1 μg of pSV40β-gal plasmid. Cells were then washed with MEM and incubated for 48 h under control conditions or under hyperoxia in medium containing 10% FBS and 8 mM glutamine. At the end of this incubation, cells were washed with cold PBS and then harvested, and cellular extracts were prepared by lysing the cells in 0.5 ml lysis solution (8 mM $MgCl_2$, 25 mM Tris pH 8, 1 mM dithiothreitol (DTT), 1% Triton X-100, and 15% glycerol). Luciferase assays were performed using an equal volume of extracts. Luciferase activity was measured immediately after addition of 100 μl substrate solution (0.5 mM ATP, 0.5 mM D-luciferin) in a luminometer (LKB, Gaithersburg, MD). Under these experimental conditions, initial studies showed that the activities measured in the cell extracts were within the linear range. Luciferase activity was then normalized for transfection efficiency as determined by β-galactosidase activity and β-galactosidase staining. For each reporter plasmid, at least three independent transfections were performed.

Expression vectors p(CMV-p50) and p(CMV-p65) for the two subunits of NF-κB transcription factor, p65 and p50, coupled to the cytomegalovirus promoter (a gift from Dr. P. Baeuerle, Tularik, San Francisco, CA) were also used for co-transfection experiments with the construct p(−1400/+12) IGFBP-2-LUC described above. For these experiments, cells were transfected using 10 μl of lipofectine reagent in 3 ml of serum and antibiotic free medium. Each 100 mm dish received 5 μg of reporter plasmid and variable amounts of expression vectors (the two expression vectors were added at the same quantities). In addition, 1 μg of β-galactosidase expression vector was used with each dish.

2.3. Nuclear extract preparation and gel mobility shift assay

2.3.1. Nuclear extract preparation

Nuclear extracts were prepared from type 2 cells by the method of Schreck et al. with minor modifications after treatment with or without O_2 [28]. After three washings in cold PBS, cells were mechanically detached, and pelleted by centrifugation at $2000\times g$ for 5 min. The cellular pellet was volumed, resus-

pended in 3 vols. of solution A (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 1% NP-40) with freshly added protease inhibitors (1 μg/ml leupeptin, 5 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride) and lysated for 5 min at 4°C in agitation. Membrane lysis was accomplished by the presence in solution A of 1% NP-40. The nuclei were then collected by centrifugation at $2000 \times g$ for 5 min and the supernatant (cytosolic extract) was stored at −80°C until analysis by electrophoretic mobility shift assays. The nuclear pellet was resuspended in 1 vol. of solution B (20 mM HEPES, 1.5 mM $MgCl_2$, 300 mM KCl, 0.5 mM DTT, 0.2 mM EDTA, 25% glycerol) with freshly added protease inhibitors as described above and agitated vigorously at 4°C for 30 min. Nuclear debris were collected by centrifugation at $15\,000 \times g$ for 30 min, and the supernatant (nuclear extract) was stored at −80°C until analysis by electrophoretic mobility shift assays. The protein concentrations were determined using the Bio-Rad Protein Assay kit (Bio-Rad, Richmond, CA).

2.3.2. Electrophoretic mobility shift assays

Electrophoretic mobility shift assays (EMSA) were performed using NF-κB probes corresponding to NF-κB binding sequences present in the rat IGFBP-2 promoter. Five different NF-κB oligonucleotides were obtained commercially (Oligo-Express, Paris) and used in the EMSA. They included the recognition sites for the four putative NF-κB sites present in the IGFBP-2 promoter [27]: NF-κB_1 (5′-TCTATGGAATCTCCAAGCTT-3′), NF-κB_2 (5′-GGGAAGGGAGACGCCTCACT-3′), NF-κB_3 (5′-AGTAGGGGGACCCGCCGAAT-3′), NF-κB_4 (5′-AAGGAGGCGTCTCCCGCGGT-3′) and a mutated NF-κB_1 sequence (5′-TCTATTTAATCTCCAAGCTT-3′). EMSA were also performed using NF-1 probe (5′-AAGAAAAACCGTTCCTACCATACTAA-3′) (a gift from Dr. De Keyzer, Cochin Hospital, Paris, France). The double-stranded probes were obtained from annealing of two synthesized oligonucleotides and end-labeled using T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP. Unincorporated nucleotides were removed by filtration through a Bio-spin column (Bio-Rad). Binding reaction was carried out in 20 μl binding mixture (20 mM HEPES, 0.1 mM EDTA, 50 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 4 mM spermidine, 2 mM DTT, 100 μg/ml albumin, 17.5% glycerol), containing 1 μg of the competitor poly(dI-dC) and 4 μg of nuclear proteins [28]. After addition of nuclear extracts, reaction was allowed for 15 min at 4°C. Thereafter, 15 000 cpm of the NF-κB probe were added and the incubation continued for 30 min at 4°C. The competition experiments were performed using increasing amounts of unlabeled specific NF-κB probe (10-, 100-, 200-fold molar excess) and non-specific poly(dI-dC) (1 μg), which were mixed with extracts before the addition of the labeled DNA probe. For supershift experiments, extracts were preincubated overnight at 4°C with 1 μg of 3 μg of monoclonal anti-NF-κB antibody (p65 subunit, Boehringer Mannheim, Germany) before the labeled DNA probe was added. Samples were fractionated by electrophoresis on 5% non-denaturing polyacrylamide gel in 1×TBE (10 mM Tris, 9 mM boric acid, 0.1 mM EDTA), which had been pre-electrophoresed for 1 h at 100 V. Gels were run at 150 V for 2.5 h. Following electrophoresis, gels were dried under vacuum at 80°C and exposed to X-ray Hyperfilm at room temperature. Results were quantified by laser scanning densitometry of the autoradiographs.

2.4. Protein studies

Cells were washed 3 times with cold PBS and lysed by addition of 200 μl lysis buffer (250 mM NaCl, 50 mM HEPES pH 7.0, 5 mM EDTA, 1 mM DTT, 0.1% nonidet NP-40, 10 μg/ml leupeptin, 10 μg/ml apoprotinin, 50 μg/ml phenylmethylsulfonyl fluoride, 2 mM sodium pyrophosphate, 1 mM sodium orthovanadate) [12]. The lysates were incubated at 4°C overnight and clarified by centrifugation at $10\,000 \times g$ for 10 min at 4°C. Equal quantities of proteins were loaded for each experimental condition and, after boiling, the samples were analyzed on SDS-PAGE (11% polyacrylamide) under reducing conditions.

Western blots were prepared by electrotransferring the proteins onto 0.45 μm nitrocellulose membranes (NC; Bio-Rad) for 1 h at 130 V. Immunoblotting was performed by first saturating the NC sheet for 4 h at room temperature in PBS containing 0.2% Tween (PBS-T) and 10% powdered milk. This was followed by incubation with diluted antiserum in 5%

milk-PBS for 20 h at 4°C. The antisera used were the following: monoclonal anti-NF-κB antibody (p65 subunit, Boehringer Mannheim), monoclonal anti-IκB-α antibody (a gift from Dr. A. Weil, Pasteur Institute, Paris, France). The membranes were then washed 3 times in PBS-T buffer and incubated for 1 h at 37°C with horseradish peroxidase conjugated goat antimouse immunoglobulin G (Amersham, UK), diluted 1/6000 in milk-PBS. The membranes were then washed 3 times in PBS-T, after which they were incubated for 1 min at room temperature in chemiluminescence reaction detection reagents (ECL Western blotting, Amersham). The membranes were then exposed to autoradiography film (Hyperfilm-ECL, Amersham).

2.5. Statistical methods

Results are reported as mean ± S.E.M. Data were analyzed using ANOVA followed, when adapted, by Bonferroni *t*-test for multiple comparisons against control conditions. Significance is assigned for $P < 0.05$.

3. Results

3.1. Activation of IGFBP-2 promoter in cells exposed to hyperoxia

To determine the effects of hyperoxia on the promoter activity of the IGFBP-2 gene, transfection experiments were performed. Type 2 cells were co-transfected with pairs of expression vectors, including one that conveys expression of luciferase gene and one that conveys expression of *E. coli lacZ* gene. One set of cells was transfected with the control pLUC promoterless construct. The second set of cells was transfected with pRSVLUC construct. The third set of cells was transfected using luciferase gene transcribed from the IGFBP-2 promoter with 1.4 kb of 5′-flanking sequence IGFBP-2: p(−1400/+12) IGFBP-2-LUC. Transfection efficiency evaluated by cell blue staining was found to fluctuate between 10 and 20%. For each set, cells were incubated under normoxia or hyperoxia for 48 h. As previously reported, exposure of type 2 cells to hyperoxia led to a rapid inhibition of cell proliferation [11,12,14]. Indeed, after 48 h O_2, no increase in cell number could be detected (data not shown). After preparation of cellular extracts luciferase and β-galactosidase activity assays were carried out. The luciferase activity in extracts of cells transfected with the promoterless construct did not exceed the apparatus background level. Using the pRSVLUC construct, no differences in luciferase activity could be observed in extracts of cells cultured under either normoxia or hyperoxia (data not shown). As reported in Fig. 1, exposure to O_2 was found to induce luciferase activity of the p(−1400/+12) IGFBP-2-LUC construct, indicating that IGFBP-2 promoter was active under hyperoxic conditions.

3.2. IGFBP-2 promoter contains functional NF-κB binding sites

Examination of the IGFBP-2 promoter revealed the presence of four sequences that match the NF-κB binding consensus: NF-κB binding consensus: GGGRNNYYCC, where R = purine, Y = pyrimidine and N = any nucleotide. These sequences in the IGFBP-2 promoter are the following: nucleotides −1305 bp to −1297 bp: GGAATCTCC; −1072 bp to −1063 bp: GGGAGACGCC; −267 bp to −258 bp: GGGGGACCCG; −73 bp to −64 bp: GGCGTCTCCC.

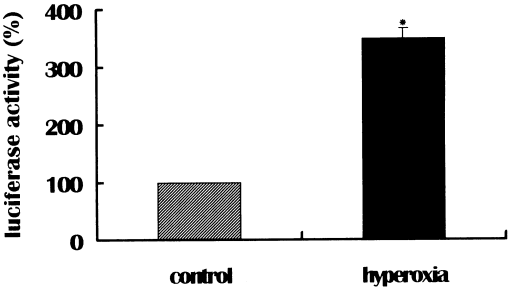

Fig. 1. Activity of IGFBP-2 promoter in type 2 cells. Type 2 cells were co-transfected with the plasmid p(−1400/+12) IGFBP-2-LUC and the plasmid conveying expression of *E. coli lacZ* gene, as described in Section 2. The cells were then cultured in either control or hyperoxia conditions (48 h under 95% O_2). Luciferase assays were performed using an equal amount of proteins and luciferase activity was then normalized for transfection efficiency, as determined by β-galactosidase activity. IGFBP-2 promoter activity values on the graph correspond to the ratio luciferase activity/β-galactosidase activity. The mean and S.E.M. for three independent experiments performed in triplicate are shown (*$P < 0.05$).

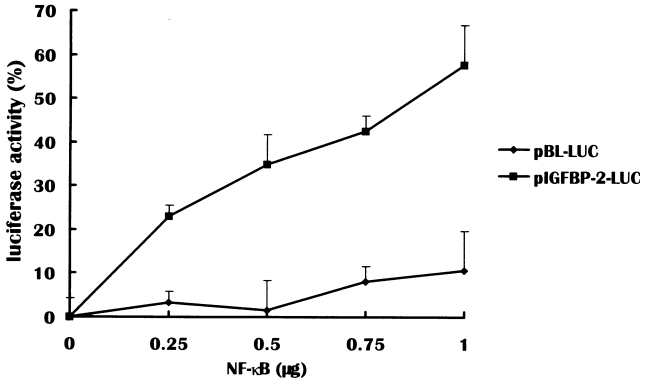

Fig. 2. NF-κB activates the IGFBP-2 promoter. p(−1400/+12) IGFBP-2-LUC or pBL-LUC constructs (5 μg) were co-transfected with increasing amounts of NF-κB expressing plasmids (pCMV-50 and pCMV-65) into type 2 cells, as described in Section 2. The luciferase values, normalized for β-galactosidase, were expressed as percentage over the activity obtained in the absence of NF-κB plasmids.

To determine whether NF-κB binding was involved in the transcriptional regulation of IGFBP-2, p(−1400/+12) IGFBP-2-LUC construct was co-transfected with increasing amounts of p50 and p65 expressing plasmids into type 2 cells cultured under control conditions. As shown in Fig. 2, expression of p50 and p65 stimulated luciferase activity. These results indicated that NF-κB was able to activate IGFBP-2 promoter in a concentration-dependent fashion and therefore suggested a role of NF-κB binding sites in the response. No changes in luciferase activity could be found when the p50 and p65 expressing plasmids were co-transfected with the promoterless firefly luciferase reporter gene pBL-LUC (Fig. 2).

3.3. *Involvement of NF-κB binding sites in the transcriptional activation of IGFBP-2 in oxidant-exposed cells*

To determine the involvement of the different putative NF-κB binding sites in the transcriptional regulation of IGFBP-2 promoter, gel mobility shift assays were performed with the four NF-κB double-stranded oligonucleotides identical to the sequences observed in the IGFBP-2 promoter. Gel retardation

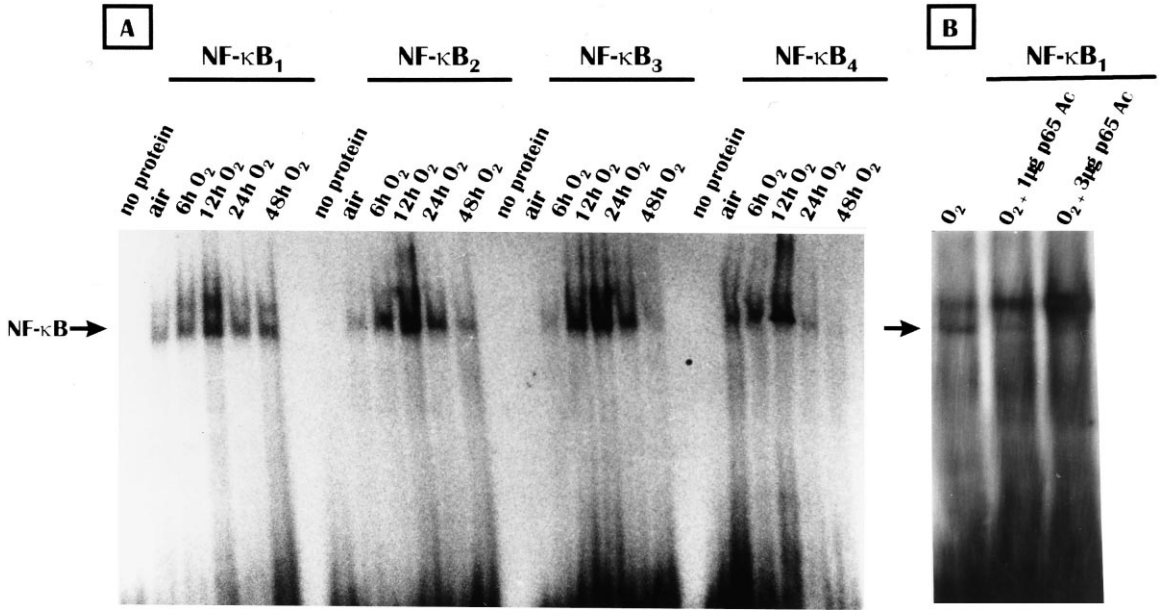

Fig. 3. Effects of O_2 on the activity of the four NF-κB sites. (A) Nuclear proteins were extracted from cells cultured under control conditions (air) or under hyperoxia (95% O_2) for 6 h, 12 h, 24 h or 48 h. 15 000 cpm of the four γ-^{32}P end-labeled NF-κB probes were incubated without protein or with 4 μg of nuclear extracts and followed by gel retardation assays as described in Section 2. The DNA-protein complexes formed are indicated by an arrow. The uncomplexed DNA probes are shown on the bottom. (B) Nuclear proteins were extracted from cells cultured under hyperoxia (95% O_2) for 12 h. 15 000 cpm of γ-^{32}P end-labeled NF-κB$_1$ probe was incubated in the presence of 4 μg of nuclear extracts without or with the monoclonal antibody anti-p65 NF-κB subunit (1 μg or 3 μg). Gel retardation assays were then performed as described in Section 2. The DNA-protein complexes formed are indicated by an arrow. The uncomplexed DNA probes are shown on the bottom.

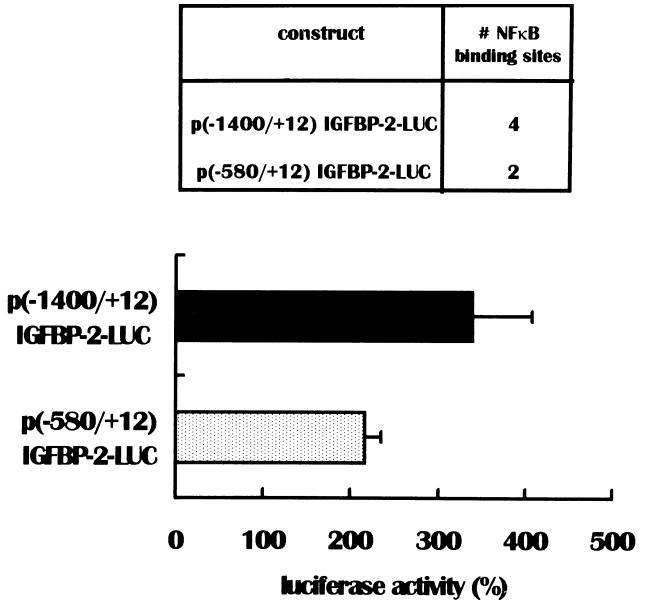

Fig. 4. Effects of hyperoxia on IGFBP-2 promoter constructs containing different numbers of NF-κB sites. Type 2 cells were co-transfected with p(−1400/+12) IGFBP-2-LUC construct containing the two putative NF-κB sites located in the distal part of the promoter (at position −1305/−1297 and −1072/−1063, respectively) and the two putative NF-κB sites located in the proximal part of the promoter (at position −267/−258 and −73/−64, respectively), or with p(−580/+12) IGFBP-2-LUC construct that contains only the two proximal NF-κB sites, in association with the plasmid conveying expression of *E. coli lacZ* gene, as described in Section 2. The cells were then cultured under either control or hyperoxic conditions (48 h O_2). Luciferase assays were performed using an equal amount of proteins and luciferase activity was normalized for transfection efficiency, as determined by β-galactosidase activity. The values obtained under hyperoxia were expressed as percentage over the activity obtained under control conditions. The mean and S.E.M. for three independent experiments performed in triplicate are shown.

analysis revealed that these oligonucleotides were able to bind nuclear factors and that the binding increased in O_2-treated cells. No difference in the binding of NF-κB nuclear factors could be detected between the different sites, the experiments being performed each time with the same nuclear extracts and run on the same gel as shown in Fig. 3A. Moreover, for the different NF-κB binding sites the maximal binding was observed in the experimental conditions where the cells were exposed to hyperoxia for 12 h. Formation of the DNA-protein complexes was further studied in the presence of antibodies directed against p65 NF-κB subunits. A supershift was observed after addition of anti-p65 antibody, with an increased intensity of the upper band in the presence of increasing amounts of antibody (Fig. 3B) [29,30].

From data reported in Fig. 3A it appeared that the activation of the four NF-κB binding sites may be similar in cells exposed to hyperoxia. To give more support to these results, co-transfection experiments were performed using p(−1400/+12) IGFBP-2-LUC construct containing the two putative NF-κB sites located in the distal part of the promoter (at position −1305/−1297 and −1072/−1063, respectively) and the two putative NF-κB sites located in the proximal part of the promoter (at position −267/−258 and −73/−64, respectively), and using p(−580/+12) IGFBP-2-LUC construct that contains only the two proximal NF-κB sites. The promoter activity of the construct containing only the two proximal NF-κB sites was still inducible by O_2 albeit at a lower level compared to the luciferase activity obtained using the p(−1400/+12) IGFBP-2-LUC construct which contains the four NF-κB sites with a 39% reduction of activity (Fig. 4). These experiments indicated that the distal and the proximal parts of the IGFBP-2 promoter, each containing two putative NF-κB binding sites, seemed to contribute to a similar extent to the effect of hyperoxic treatment on IGFBP-2 promoter activity.

To confirm activation of NF-κB in oxidant-exposed cells, the specificity of gel-shift complexes was assessed using competition experiments. When nuclear proteins were incubated in the presence of a 10–200-fold molar excess of unlabeled oligonucleotides, the binding was competitively blocked, whereas incubation with unlabeled oligonucleotides corresponding to the irrelevant sequence poly(dI-dC) did not modify formation of the complexes. In the results shown in Fig. 5A, experiments were performed using oligonucleotides corresponding to NF-$κB_1$. Similar results were obtained using oligonucleotides corresponding to the other putative binding sites (data not shown).

To verify that DNA requirements for binding of factors to the region of the IGFBP-2 promoter defined by the NF-κB oligonucleotide were similar to those exhibited by members of the NFκB family, a double-strand oligonucleotide that incorporated a 2-base pair mutation in the NF-$κB_1$ oligonucleotide

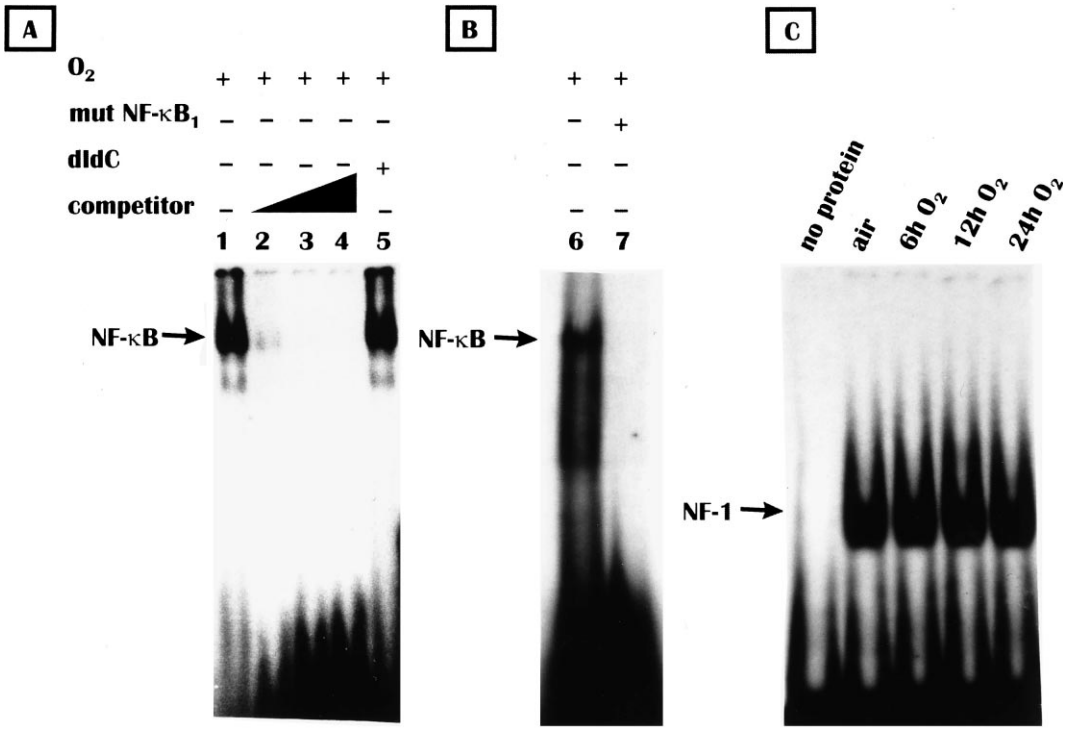

Fig. 5. Gel mobility shift assays using NF-κB or NF-1 probes: competition experiments and O_2 specificity studies. (A,B) Nuclear proteins were extracted from cells cultured under hyperoxia (95% O_2). 15 000 cpm of γ-^{32}P NF-κB_1 (lanes 1–6) or Mut NF-κB_1 (lane 7) probe were incubated with 4 μg of nuclear extracts with increasing amounts of unlabeled NF-κB probe (10-, 100-, 200-fold molar excess) (lanes 2–4) or poly(dI-dC) (lane 5) as competitors and followed by gel retardation assays as described in Section 2. The DNA-protein complexes formed are indicated by an arrow. The uncomplexed DNA probes are shown on the bottom. Similar results were obtained upon repetition of the experiment. (C) Nuclear proteins were extracted from cells cultured under control conditions (air) or under hyperoxia (95% O_2) for 6 h, 12 h, or 24 h. 15 000 cpm of the γ-^{32}P end-labeled NF-1 probe was incubated without protein or with 4 μg of nuclear extracts and followed by gel retardation assays as described in Section 2. The DNA-protein complexes formed are indicated by an arrow. The uncomplexed DNA probes are shown on the bottom. Similar results were obtained upon repetition of the experiment.

was synthesized: NF-κB_1 (5′-TCTATGGAATCTC-CAAGCTT-3′) was changed to (5′-TCTATTTA-ATCTCCAAGCTT-3′). This GG to TT mutation abolishes binding of an NF-κB-like factor to the major histocompatibility class II-associated invariant chain gene [31]. When this mutant oligonucleotide, Mut NF-κB_1, was radiolabeled and used in gel mobility shift assay, it failed to bind to nuclear proteins as compared with the wild type NF-κB_1 sequence (Fig. 5B). Thus a mutation known to inhibit the binding of NF-κB_1 inhibits the ability of nuclear extracts to bind to a target sequence in the IGFBP-2 promoter.

To further confirm the specificity of activation of NF-κB by oxidant, gel shift was performed using an oligonucleotide to which binding is invariant. For this experiment, we used the ubiquitous nuclear factor 1 (NF-1) oligonucleotides [32,33]. Gel retardation analysis revealed that the binding was similar in the nuclear extracts obtained from cells cultured under normoxia or under hyperoxia for various durations (Fig. 5C).

3.4. Effect of hyperoxia on NF-κB protein

To further substantiate a role for NF-κB in the transcriptional activation of IGFBP-2 in oxidant-exposed cells, NF-κB protein level was studied. Nuclear extracts were prepared from exponentially growing cells and from cells exposed to hyperoxia and analyzed by immunoblotting with anti-p65 antibody. As shown in Fig. 6A, exposure to hyperoxia led to a rapid accumulation of p65 in the nuclear extracts. This was observed after only 6 h of O_2

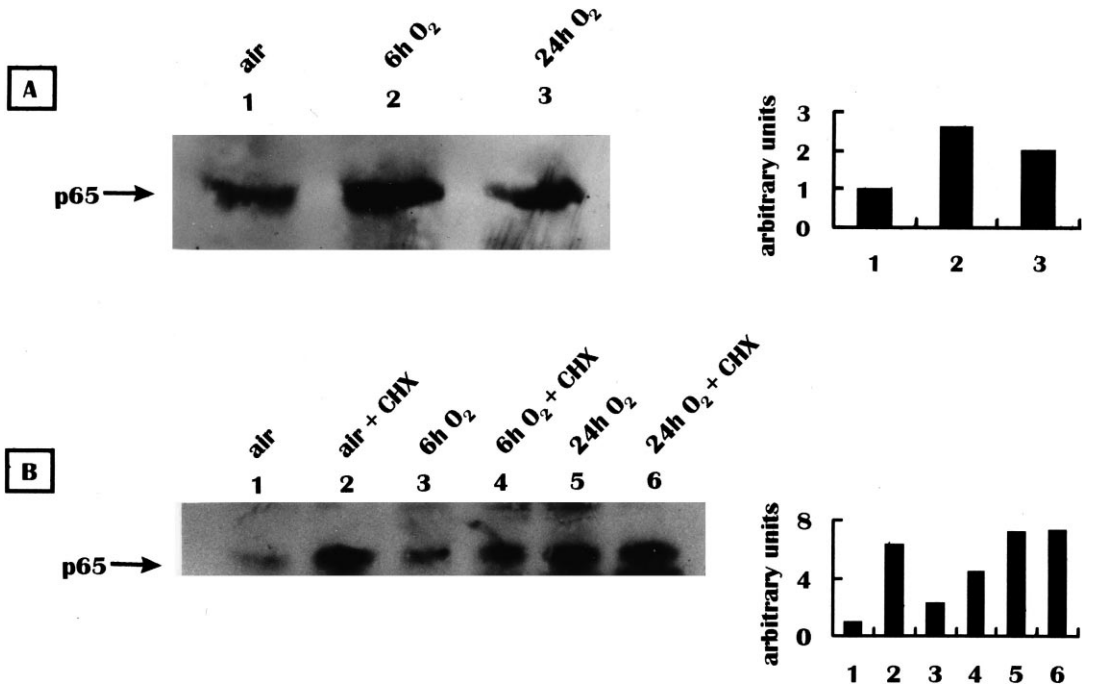

Fig. 6. Effects of O_2 and cycloheximide on nuclear NF-κB protein level. (A) Nuclear proteins were extracted from cells cultured under air (control condition) or under hyperoxia (95% O_2) for 6 h or 24 h, and analyzed by immunoblotting. Proteins were fractionated on SDS-PAGE, transferred to nitrocellulose, and probed with the monoclonal antibody to the p65 subunit of NF-κB, as described in Section 2. An autoradiogram of signals for the p65 subunit of NF-κB (65 kDa) is shown. The histogram shows a quantitative representation of hybridization signals obtained from laser densitometric analysis of the exposed films. Results are expressed in arbitrary densitometric units after normalization on the basis of control condition signal. (B) Proteins were extracted from cells cultured under air (control condition), or under hyperoxia (95% O_2) for 6 h or 24 h, in the absence or presence of cycloheximide (CHX). Proteins were analyzed by immunoblotting. Samples were fractionated on SDS-PAGE, transferred to nitrocellulose, and probed with the monoclonal antibody to the p65 subunit of NF-κB, as described in Section 2. An autoradiogram of signals for the p65 subunit of NF-κB (65 kDa) is shown. The histogram shows a quantitative representation of hybridization signals obtained from laser densitometric analysis of the exposed films. Results are expressed in arbitrary densitometric units after normalization on the basis of control condition signal.

treatment, confirming that the NF-κB factor was rapidly translocated from the cytoplasm to the nucleus of type 2 cells cultured under hyperoxia.

To determine whether accumulation of NF-κB in oxidant-treated cells involved de novo protein synthesis, cellular protein extracts were prepared from control or O_2-exposed cells cultured in the presence of an inhibitor of protein synthesis, cycloheximide (10 μg/ml) for 6 h, and analyzed by immunoblotting with anti-p65 antibody. Results are shown in Fig. 6B. Exposure to oxidant was associated with increased levels of p65 compared to the levels observed in exponentially growing cells, and addition of cycloheximide was not associated with a decrease in the amounts of p65. These results indicate that oxidant-induced activation of NF-κB did not involve de novo NF-κB protein synthesis. This is further supported by the results obtained in control cells showing a low level of p65 in conditions when cells were cultured without protein synthesis inhibitor and a dramatic induction upon treatment with cycloheximide.

Further evidence for the role of NF-κB in the transcriptional regulation of the IGFBP-2 promoter by oxidants was provided by the use of PDTC [34,35]. PDTC is a molecule which can suppress radical production by chelating metal very efficiently. We show in Fig. 7 that the level of nuclear protein p65 decreased in the presence of PDTC in a dose-dependent manner, indicating that PDTC was able to inhibit the effects of hyperoxia on NF-κB activation. In addition, gel shift performed using NF-$κB_1$ probe and nuclear extracts from cells cultured under hyperoxia

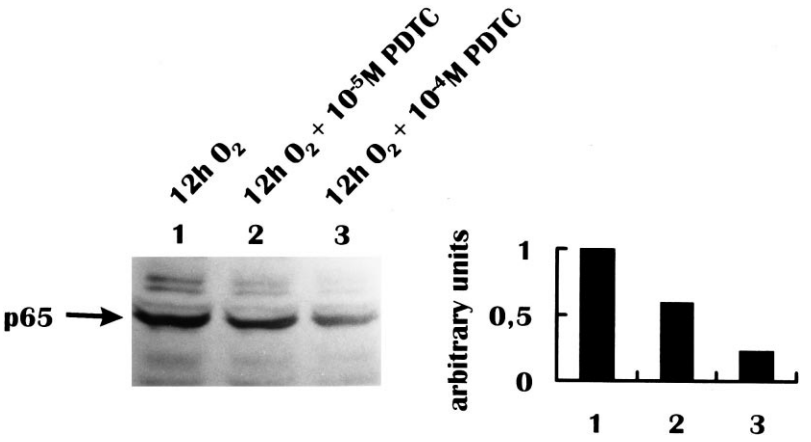

Fig. 7. Effects of pyrrolidine dithiocarbamate (PDTC) on nuclear NF-κB protein level. Proteins were extracted from cells cultured under hyperoxia (95% O_2) for 12 h, without or with 10^{-5} M or 10^{-4} M PDTC. Proteins were analyzed by immunoblotting. Samples were fractionated on SDS-PAGE, transferred to nitrocellulose, and probed with the monoclonal antibody anti-p65 NF-κB subunit, as described in Section 2. An autoradiogram of signals for the p65 subunit of NF-κB (65 kDa) is shown. The histogram shows a quantitative representation of hybridization signals obtained from laser densitometric analysis of the exposed films. Results are expressed in arbitrary densitometric units after normalization on the basis of condition without PDTC signal.

for 12 h with or without PDTC indicated a decreased binding in PDTC-treated cells (data not shown).

The increased accumulation of NF-κB in the nuclear fraction of O_2-exposed cells led to suggest an altered regulation of IκB with an increased degradation by oxidants. To test this hypothesis, abundance of IκB-α was evaluated in the cytosolic extracts of cells cultured under either control conditions or hyperoxia for 6 and 24 h. Results are presented in Fig. 8. Exposure to oxidants was associated with a decrease in the abundance of IκB-α, and this was dependent on O_2 treatment duration with a maximal effect at 24 h.

4. Discussion

In the present study, we have shown that oxidant exposure of lung alveolar epithelial cells induced transcriptional activation of IGFBP-2, a gene whose expression has been reported to be associated with inhibition of type 2 cell proliferation. Study of the mechanisms involved provided evidence that NF-κB may play a role in this process. Indeed, data presented herein demonstrated that NF-κB activated IGFBP-2 promoter in transient transfection assays, and that NF-κB was activated in O_2-treated cells. Moreover, gel shift analysis documented an increased

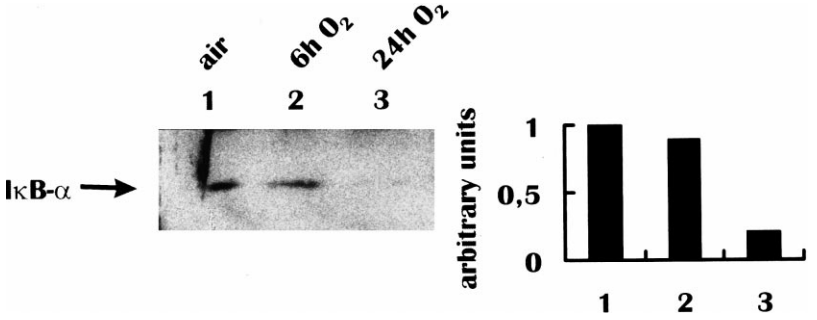

Fig. 8. Effects of O_2 on IκB protein level. Proteins were extracted from cells cultured under air (control condition), or under hyperoxia (95% O_2) for 6 h or 24 h. Proteins were analyzed by immunoblotting with anti-IκB-α antibody, as described in Section 2. An autoradiogram of signals for IκB-α (37 kDa) protein is shown. The histogram shows a quantitative representation of hybridization signals obtained from laser densitometric analysis of the exposed films. Results are expressed in arbitrary densitometric units after normalization on the basis of control condition signal.

binding to the NF-κB binding sites present in the IGFBP-2 promoter in lung cells growth-arrested by oxidants.

Our present results extend previous studies on the mechanisms involved in the growth control of lung alveolar epithelial cells. Using various modes of block of type 2 cell proliferation including serum deprivation and O_2 treatment, we have shown that inhibition of DNA synthesis observed under these experimental conditions was associated with similar induction of IGFBP-2, and that control of IGFBP-2 expression was exerted mostly at the level of transcription [14–16]. IGFBP-2 is a member of the IGF system which is known to play an important role during the late part of the G_1 phase of the cell cycle [36]. Taken together, these data support the hypothesis that common regulatory mechanisms are involved in the growth arrest of lung epithelial cells induced under various conditions, and suggest that events operating late in the G_1 phase of the cell cycle play a key role in the regulation of type 2 cell proliferation. To gain further insights into these mechanisms, we focused on the factors which may participate in the activation of IGFBP-2 promoter. From analysis of the genomic sequence upstream from the transcriptional start of IGFBP-2 which revealed the presence of consensus binding sites for NF-κB, a possibility has emerged that modulation of binding of NF-κB to its cognate element in the IGFBP-2 promoter may control lung alveolar epithelial cell proliferation. This hypothesis was sustained by the current knowledge on the processes involved in the activation of NF-κB. It is now well established that NF-κB is an oxidative stress responsive transcription factor, and that reactive O_2 species play a key role in its activation [20]. Moreover, the activation of NF-κB has been shown to be blocked by a variety of antioxidant components [35,37]. Oxidative injury which results from an imbalance in oxidant-antioxidant equilibrium in favor of oxidants can occur in situations associated with high production of reactive O_2 species, such as hyperoxia [38]. It can also be observed in a number of stress situations including culture without serum. Indeed, an alteration in the level of antioxidants and mainly of the glutathione system has been documented in these conditions [39,40]. Interestingly, Messina et al. indicated that lymphocytes depleted of glutathione were blocked in cell cycle progression before entry into S phase suggesting a link between glutathione depletion and cellular growth arrest late in G_1 [41]. These various studies give support to the hypothesis that common mechanisms associated with the block of proliferation of lung alveolar epithelial cells exposed to oxidants or to other situations such as serum deprivation may involve oxidative stress and may participate in the activation of NF-κB [42].

The IGFBP-2 gene contains four NF-κB binding sites in the 5′-flanking region, and data reported herein indicated that these sites were active under O_2 exposure, suggesting that NF-κB may be involved in IGFBP-2 regulation. The observation that IGFBP-2 promoter constituted a target for the action of NF-κB was first supported by the co-transfection experiments using p50 and p65 expression vectors. A role of NF-κB in the transcriptional control of IGFBP-2 in O_2-exposed cells was then documented using gel retardation assays; the four NF-κB sites in the IGFBP-2 promoter seemed to be activated to a similar extent upon exposure to oxidants. The precise involvement of the four putative NF-κB binding sites is now under investigation.

The transcriptional activation of IGFBP-2 was found to be associated with accumulation of NF-κB in O_2-treated cells, and results of the experiments using cycloheximide argued against de novo protein synthesis. These findings are consistent with the current understanding of NF-κB activation. The mechanisms involved associate signal-dependent phosphorylation and ubiquitination of IκB at N-terminal sites, which target the inhibitor for degradation by the 26S proteasome. In turn, this proteolytic event exposes subcellular localization motifs on NF-κB that direct it to the nuclear compartment [43,44]. Data reported herein are consistent with an increased translocation of NF-κB to the nucleus leading to an accumulation of NF-κB in the nuclear extracts of cells exposed to hyperoxia. This is sustained by the decrease in the abundance of IκB-α found in the cytosolic extracts of O_2-treated cells. Additional evidence of NF-κB activation in oxidant-exposed cells is supported by the results of experiments using PDTC. Although the mode of action of PDTC remains largely discussed, its effect on NF-κB activation has been reported in several studies to be suppressive [43,44]. In their report, Brennan et al.

showed that it inhibited NF-κB activation in three cell lines only at a concentration of 1 mM [43,44]. In the present study, decrease in the levels of nuclear p65 was observed in the presence of 10 μM PDTC.

The present results support a role for NF-κB in induction of IGFBP-2 gene in lung alveolar epithelial cells growth-arrested by oxidants. From these findings, several comments should be made. First, in most studies NF-κB has been shown to be involved in the regulation of cytokine genes; therefore data reported herein are consistent with an action of NF-κB in the control of a large variety of genes. Second, if NF-κB could participate in the activation of IGFBP-2, it is likely that this is not the only one factor involved and that other mechanisms are implicated. One critical question which needs to be addressed is the role of these molecules in the control of lung alveolar type 2 cell proliferation and how they may be linked to the key regulators of the cell cycle machinery which control entry into S phase. These regulators include the cyclins and their associated kinases, the cyclin-dependent kinases (CDKs) [45,46]. Activities of the various G_1 CDKs are regulated by a number of factors including the CDK inhibitors (CKIs) [47]. In previous studies, we have shown that O_2 blocked entry of lung epithelial cells into S phase by inactivation of cyclin E-CDK2 complexes, which was associated with a dramatic induction of the CKI $p21^{CIP1}$ [12]. Recent works suggest interesting possibilities to link NF-κB to the cell cycle machinery. First, Yang et al. indicated that $p21^{CIP1}$ could increase NF-κB activation [48]. This is in agreement with data reported by Siebenlist showing that increased expression of c-Rel favored growth arrest [22]. This growth arrest was correlated with inhibition of CDK2 and CDK4 apparently through transcriptional activation of $p21^{CIP1}$ by c-Rel. Recently, Perkins et al. provided some insights into the mechanisms by which NF-kB and $p21^{CIP}$ can be associated with growth arrest [49]. They demonstrated interactions between the transcriptional activation domain of p65 and the coactivator p300. This interaction appeared to occur in the amino-terminal region of p300 distinct from a carboxyl-terminal region of p300 required for the binding to the cyclin E-CDK2 complex. This led them to propose that $p21^{CIP1}$, by interacting with cyclin E-CDK2, may modulate the interactions of NF-κB and CDK complexes, thus contributing to coordinate transcriptional activation by NF-κB of growth-related genes and cell cycle progression. These data provide support to link increase in NF-κB activity, increase in $p21^{CIP1}$, and growth arrest in lung epithelial cells exposed to oxidants.

In the past few years much attention has been focused on the involvement of IGFBPs in growth control. In lung epithelial cells IGFBP-2, which is the predominant form of IGFBPs produced as determined by ligand blotting experiments, has been shown to be associated with block of proliferation. Similar findings were reported by Park et al. in intestinal epithelial cells [50]. In other cell systems, IGFBP-3 was found to be the main IGFBP produced and to be associated with growth inhibition [51]. If these various studies have helped to establish the involvement of IGFBPs in the control of cell proliferation through either an IGF-dependent or IGF-independent signaling pathway, it remains now to understand how IGFBPs can be linked to the cell cycle machinery and to determine the mechanisms regulating their expression. At the present time, there are no reports in the literature on the role of NF-κB in the transcriptional activation of IGFBPs. The involvement of another factor known to play a central role in late G_1 checkpoint and to act as growth suppressor, the protein p53, has recently been discussed. In colon carcinoma cells, Buckbinder et al. have provided data documenting induction of IGFBP-3 in growth-arrested cells and demonstrating transcriptional regulation of IGFBP-3 by p53 [52]. These findings support a role for IGFBP-3 in mediating inhibitory actions of p53 on cell proliferation. Interestingly, a link between NF-κB and p53 has been reported by Wu et al. [53]. These authors produced direct evidence that NF-κB specifically recognized the NF-κB sites in the p53 promoter and activated p53. From these various studies, it may be hypothesized that NF-κB could regulate expression of several IGFBPs either directly or indirectly through p53.

To conclude, the data reported herein provide evidence that NF-κB may play a role in the transcriptional regulation of IGFBP-2 in lung alveolar epithelial cells exposed to oxidants. We speculate that signaling events involving accumulation of NF-κB levels converge to activate IGFBP-2 in situations as-

sociated with an imbalance in oxidant-antioxidant equilibrium and block of proliferation. Dissection of these signaling pathways should provide insights into the mechanisms involved in cell cycle regulation of IGFBP-2 and into the role of IGFBP-2 in the control of lung epithelial cell proliferation.

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