

# Mechanisms of Synergistic Cytokine-Induced Nitric Oxide Production in Human Alveolar Epithelial Cells

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**Nitric oxide (NO) derived from inducible NO synthase (iNOS) at sites of inflammation is closely related to host defense against infection and airway inflammation. Cytokines are known to stimulate NO production in human alveolar epithelial cells in a synergistic (nonlinear or nonadditive) manner. The mechanism of this synergy is not known. We measured the activation of the transcription factor NF- $\kappa$ B, the iNOS protein, and NO production in A549 monolayers (human alveolar epithelial cell line) in response to different combinations of IL-1 $\beta$ , INF- $\gamma$ , and TNF- $\alpha$  (100 ng/ml), and the cofactors FMN, FAD, and BH4. We found that both IL-1 $\beta$  and TNF- $\alpha$  could independently activate cytosolic NF- $\kappa$ B, direct its translocation into the nucleus, and induce iNOS monomer synthesis. In addition, different combinations of cytokines produced synergistic amounts of iNOS monomers. Exogenous BH4 (0.1  $\mu$ M) had no impact on NO production induced by cytokine combinations that included IL-1 $\beta$ , but significantly enhanced NO production in the presence of INF- $\gamma$  and TNF- $\alpha$ , and allowed TNF- $\alpha$  independently to produce NO. We conclude that there are at least three mechanisms of synergistic cytokine-induced NO production: (1) the biosynthesis of iNOS monomer due to nonlinear interactions by transcription factors, (2) synergistic cytosolic activation of NF- $\kappa$ B, and (3) parallel biosynthesis of BH4 in the presence of cytokine combinations that include IL-1 $\beta$ .** © 2001 Elsevier Science

**Key Words:** iNOS; A549; tetrahydrobiopterin; cyto-mix.

Nitric oxide (NO), a simple diatomic free radical, acts as a natural anticoagulant, vasodilator, neurotransmitter, and mediator of immune system function (1–3). NO plays an important role in the physiological regulation of normal airway function, and is released through the intermittent catalytic action of constitutive NO synthase (cNOS). In addition, large transient production of NO at sites of inflammation is derived from inducible NO synthase (iNOS) and is closely related to host defense against infection (4). There is currently considerable interest in inhibiting NO production as a therapeutic intervention in inflammatory diseases (5, 6); hence, it is crucial to understand the mechanisms underlying its production.

The transcription of iNOS is of particular importance in the lungs including its regulation by three cytokines—tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ), and interferon- $\gamma$  (INF- $\gamma$ ) (5, 7, 8). NO production by iNOS in response to TNF- $\alpha$ , IL-1 $\beta$ , and INF- $\gamma$  has been widely studied in several different species and many different pulmonary cells, including vascular smooth muscle cells, bronchial smooth muscle cells, fibroblasts, bronchial epithelial cells, and alveolar epithelial cells (9, 10). The rate of NO production by these cells in response to different combinations of TNF- $\alpha$ , IL-1 $\beta$ , and INF- $\gamma$  is synergistic. The synergistic response is unique to

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cell type and species (11). We have recently reported a detailed investigation of the synergistic NO production by A549 cells (human alveolar epithelial cell line) to TNF- $\alpha$ , IL-1 $\beta$ , and INF- $\gamma$  (12). Although the mechanism of iNOS activation by individual cytokines has been reported, the mechanism underlying the synergy in NO production is poorly understood.

The mechanism underlying the activation of iNOS for the individual cytokines follows similar paths. A reservoir of inactive cytosolic transcription factors are activated following the binding of a cell surface membrane and a cascade of protein phosphorylations (5, 13), which causes the transcription factors to translocate to the nuclear compartment. In the case of IL-1 $\beta$ , the key transcription factor is nuclear factor- $\kappa$ B (NF- $\kappa$ B) (14), and in the case of INF- $\gamma$ , the transcription factor is IRF-1 (5). Once present in the nuclear compartment, the transcription factors bind to multiple cognate sequences on several inflammatory genes including the gene that encodes the iNOS monomer (5, 15, 16). The fully active iNOS protein is dimeric in nature, consisting of two iNOS monomers. The functional dimer is assembled in the cytosol, and requires several cofactors (prosthetic groups) for its activity, namely, FAD, FMN, heme, and BH4 (17, 18). BH4 and heme are required for dimerization and subsequent activity (19).

The goal of our current study is to investigate the mechanism of synergy among TNF- $\alpha$ , IL-1 $\beta$ , or INF- $\gamma$ . Based on the current understanding of iNOS induction, we proposed the following testable hypotheses, which are not necessarily exclusive, to begin an understanding of the mechanisms underlying the synergistic response to TNF- $\alpha$ , IL-1 $\beta$ , and INF- $\gamma$  in A549 cells: (1) cell exposure to TNF- $\alpha$  leads to an increased cytosolic concentration of NF- $\kappa$ B-I $\kappa$ B inactive complex, which can augment IL-1 $\beta$ -induced NO production; (2) cell exposure to combinations of cytokines results in larger nuclear concentrations of transcription factors that augment iNOS mRNA transcription and subsequent translation; and (3) cell exposure to any of the individual cytokines TNF- $\alpha$ , IL-1 $\beta$ , or INF- $\gamma$  leads to iNOS monomer production, but only IL-1 $\beta$  and INF- $\gamma$  exposure also leads to parallel biosynthesis of the cofactors necessary to form the functional iNOS dimer. Our results lead us to reject the first hypothesis and accept the second and third hypotheses. In particular, we have

identified synergistic iNOS monomer production and the parallel biosynthesis of BH4 as key mechanisms underlying the synergistic response between IL-1 $\beta$ , INF- $\gamma$ , and TNF- $\alpha$  in A549 cells.

## MATERIALS AND METHODS

*Materials.* Dulbecco's modified eagle's medium (DMEM), Dulbecco's phosphate-buffered saline (DPBS), Trypsin-EDTA solution, antibiotics (penicillin-streptomycin, amphotericin B), recombinant human interleukin-1 $\beta$  (IL-1 $\beta$ ), recombinant human tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and recombinant human interferon- $\gamma$  (INF- $\gamma$ ) were obtained from Sigma Chemical (St. Louis, MO). Fetal bovine serum was purchased from Hyclone Laboratory (Logan, UT). L-Glutamine was obtained from ICN Biochemicals (Cleveland, OH), and 48-well plates were purchased from Corning Costar (Cambridge, MA). Primary antibodies to I $\kappa$ B and NF- $\kappa$ B were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and the primary antibody to human iNOS was a generous gift of Dr. John L Humes (Merck and Co., Rathway, NJ).

*Cell culture and exposure to cytokines and cofactors.* Immortal Type II alveolar cell line of human origin (A549) were obtained from the American Type Culture Collection (ATCC). A549 is epithelial-like in morphology and originates from a human lung carcinoma. The cells were seeded at  $4 \times 10^4$  cells/cm<sup>2</sup> onto 48-well plates (Corning Costar) containing 0.5 ml of DMEM supplemented with 10% FBS, L-glutamine (2 mM), amphotericin B (5.6 mg/L), and penicillin-streptomycin (100 U/ml), and allowed to grow to confluency. Once confluent, cells were incubated in serum-free media for 24 h before exposure to cytokine(s). The cells were then exposed to cytokine(s) in serum-free media for 24 h. Following cytokine exposure, serum was returned to the culture media. The cells were exposed to different combinations of three cytokines (IL-1 $\beta$ , TNF- $\alpha$ , and INF- $\gamma$ ) at a concentration of 100 ng/ml, as well as different concentrations of the following cofactors and substrate necessary for a functional iNOS: flavin mononucleotide (FMN), flavin adenonucleotide (FAD), tetrahydrobiopterin (BH4), and L-Arginine. We then monitored the concentrations of I $\kappa$ B, NF- $\kappa$ B (cytosolic and nuclear), iNOS monomer, and

NO as a function of time (2–96 h depending on the end-product of interest).

**Preparation of cytosolic and nuclear extracts.** Cytosolic and nuclear extracts were obtained from cells following cytokine exposure for 2, 4, 6, or 24 h. All extraction procedures were performed on ice with ice-cold reagents. Cells were washed twice with DPBS, harvested by scraping into 4 ml of DPBS, and centrifuged (500g, 5 min). The pellet was dispersed in 1 packed cell volume of hypo-osmotic buffer (10 mM Hepes-KOH, pH 7.9, 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, aprotinin, pepstatin, and leupeptin, each 2 μg/ml). After 15 min on ice, Nonidet P-40 was added to a final concentration of 0.6% (v/v), and nuclei were pelleted by centrifugation (5000g, 5 min). Supernatants containing cytosolic proteins were stored at –80°C. The pelleted nuclei were dispersed in a high salt buffer (20 mM Hepes-KOH, pH 7.9, 420 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 25% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, aprotinin, pepstatin, and leupeptin, each 2 μg/ml) to solubilize DNA-binding proteins. The suspended nuclei were gently shaken for 30 min at –4°C and centrifuged in a microcentrifuge (12,000g, 20 min). The clear supernatants containing nuclear proteins were stored in small portions at –80°C until used.

**Western blot analysis.** Protein samples were mixed with an equal volume of 2× SDS sample buffer, boiled for 5 min, and separated by SDS-polyacrylamide gel electrophoresis in 7.5% gels. After electrophoresis and electrophoretic transfer of proteins to PVDF membrane (Bio-Rad) using the Mini Trans-Blot Cell (Bio-Rad), the membranes were incubated overnight in 5% nonfat milk, rinsed, and incubated for 2 h at room temperature with primary antibodies at dilutions of 1:40,000 (iNOS, Merck, NJ) and 1:500 (NFκB p65 and IκBα, Santa Cruz, CA) for rabbit polyclonal antibodies in PBS containing 0.05% Tween 20 (TPBS) with 3% nonfat milk. Excess primary antibody was removed by washing five times in TPBS. Peroxidase-labeled secondary antibodies were added at a dilution of 1:3000 (anti-rabbit IgG, Santa Cruz, CA). To assure consistency in our analysis, we utilized the same batch of antibodies for all of the experiments. After 1 h at

room temperature and five washes in TPBS, blots were incubated in enhanced chemiluminescence reagent (ECL; Amersham Pharmacia Biotech) and exposed to the film. The film was subsequently scanned with an Image Analyzer 697 (Bio-Rad) to determine the relative (background always subtracted) optical density of the blot and achieve a semi-quantitative assessment of protein concentration (see Data Analysis below).

**Nitric oxide assay.** NO activity was analyzed by chemiluminescence (Model 280 NOA, Sievers Inc., Boulder, CO). NO is unstable in the presence of oxygen, and is rapidly converted into NO<sub>2</sub><sup>–</sup> and NO<sub>3</sub><sup>–</sup> in liquid media. In order to detect NO, both NO<sub>2</sub><sup>–</sup> and NO<sub>3</sub><sup>–</sup> were converted into NO using a reducing agent (vanadium (III) chloride). To achieve high conversion efficiency, the reduction is performed at 90°C.

**Data analysis.** All data is presented as relative to baseline (time 0), and is then normalized by the maximum change from baseline within a given set of experiments (i.e., all of the NF-κB data). For example, in all of experiments in which we determined the concentration of NF-κB, we subtract the concentration at time zero and then normalize this concentration difference from baseline by the maximum concentration difference from baseline for any of the NF-κB experiments. This can be expressed as:

$$F_{i,1} = \frac{D_i(t) - D_i(0)}{(D_i(t) - D_i(0))_{i,\max}}, \quad [1]$$

where the subscripts “i” and “1” refer to the protein (iκB, NF-κB, or iNOS) and cytokine (IL-1β, INF-γ, and TNF-α), respectively, D is the density of the Western blot (see below), and the subscript “max” refers to the maximum value for protein “i.”

The concentrations of each protein were determined from the density of the Western blot. To make this semiquantitative analysis, we performed a serial dilution of the most concentrated sample (based on density) to just below the minimum density value observed. For the case of NF-κB and IκB, the response was linear (data not shown), but was nonlinear in the case of iNOS. The response of the iNOS protein followed a power-law relationship (data not shown), and the density, D, was converted to F using the following relationship:

$$F_{\text{iNOS},1} = \frac{(D_{\text{iNOS}}(t))^{0.41} - (D_{\text{iNOS}}(0))^{0.41}}{((D_{\text{iNOS}}(t))^{0.41} - (D_{\text{iNOS}}(0))^{0.41})_{\text{iNOS},1,\text{max}}}, \quad [2]$$

where the exponent of 0.41 was determined using nonlinear least squares ( $R^2 = 0.97$ ).

To determine whether synergy existed between the cytokines, we compared our observed experimental values to a predicted value based on a simple linear additive model. Two-way predicted values,  $F^{\text{P,II}}$ , are defined as the sum of the response of the two individual cytokines present alone, and the synergistic effect,  $S_{ij}$  is the difference between the observed and predicted:

$$F_{i,lm}^{\text{P,II}} = F_{i,l} + F_{i,m} \quad [3]$$

$$S_{i,lm}^{\text{II}} = F_{i,lm} - F_{i,lm}^{\text{P,II}}, \quad [4]$$

where the subscript “l” or “m” represent any two of the three cytokines. Three-way predicted values,  $F^{\text{P,III}}$ , are defined as the sum of the response of the individual and two-way synergy, and the synergistic effect,  $S_{ij}$  is the difference between the observed and predicted in the following fashion:

$$F_i^{\text{P,III}} = F_{i,l} + F_{i,m} + F_{i,n} + S_{i,lm}^{\text{II}} + S_{i,mn}^{\text{II}} + S_{i,ln}^{\text{II}} \quad [5]$$

$$S_i^{\text{III}} = F_i - F_i^{\text{P,III}}, \quad [6]$$

where the subscript refers to the protein, and the second subscript has been removed as three-way response implies all three cytokines are present.

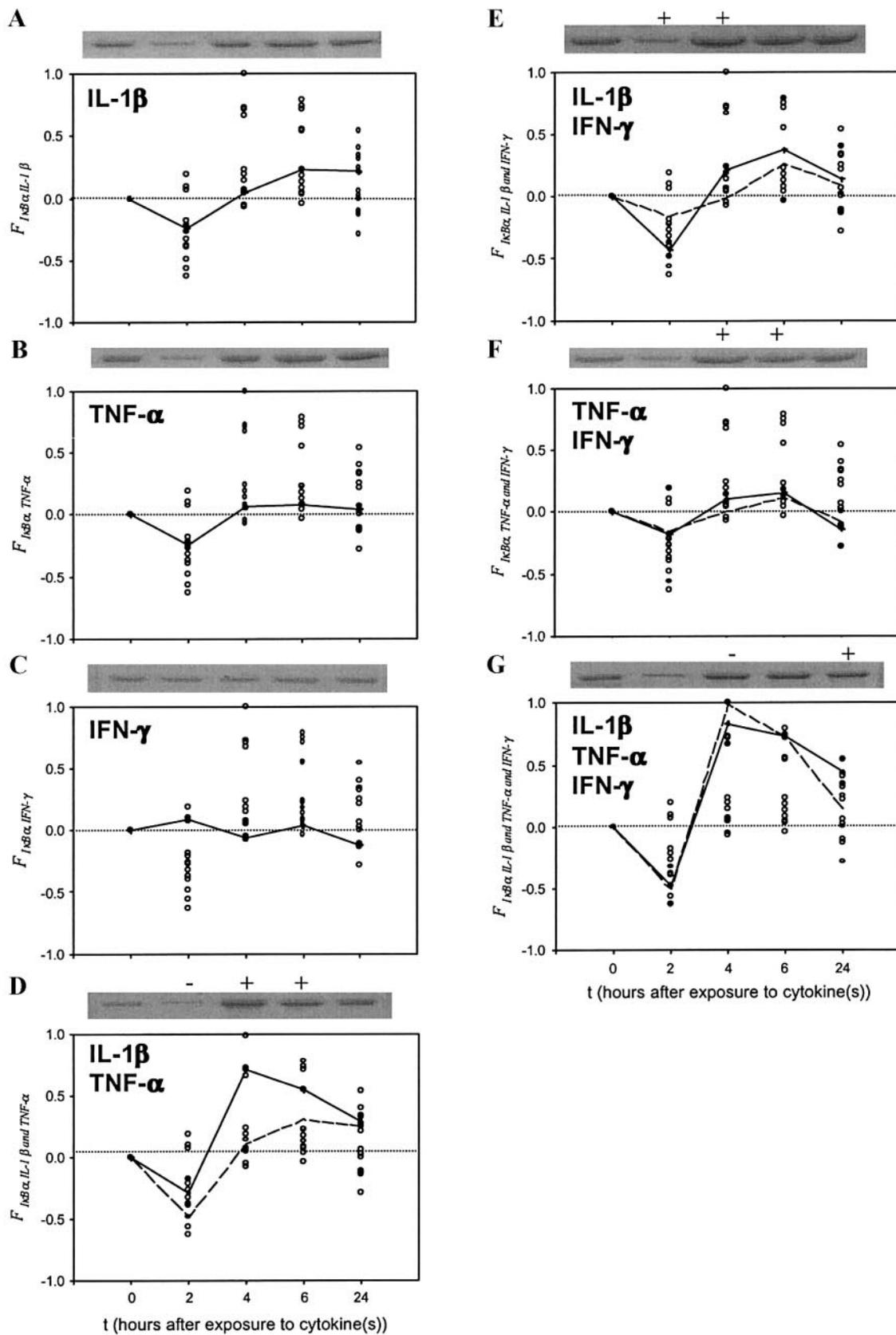
**Statistical analysis.** Like many other lab experiments, this study resulted in a small number of observations (2) for each condition. The scarcity of data and the complexity of the research hypothesis combined to make this analysis statistically challenging. In each of the experiments, our primary interest was to determine whether cytokine combinations produced a synergistic (nonlinear, nonadditive) amount of protein at different points in time. Thus, for each protein and each cytokine combination, we considered all combinations of measurements taken at each time point. For example, for the iNOS monomer, and the cytokine combination of

IL-1 $\beta$  and TNF- $\alpha$ , we would calculate all combinations (2 measurements of iNOS concentration  $\times$  2 measurements of IL-1 $\beta$  alone  $\times$  2 measurements of TNF- $\alpha$  alone = 8 combinations) of the sum of individual cytokine measurements, and subtract that sum from the measured concentration by IL-1 $\beta$  and TNF- $\alpha$  together. We then used a sign test on this difference score to identify those time point(s) where the synergy showed an improbable directional effect, and used a *t* test to detect whether the synergy magnitude was also unlikely (20). Because we realize that we are violating some test assumptions, notably that of independence of observations, we nonetheless feel that this analysis provides the reader with the best possible assessment of the research question in view of the small number of experimental measurements.

## RESULTS

**Activation of NF- $\kappa$ B following exposure of cytokine(s).** NF- $\kappa$ B exists in the cytosol as an inactive complex, NF- $\kappa$ B-I $\kappa$ B. Upon cytokine exposure, I $\kappa$ B is phosphorylated and rapidly degraded by intracellular proteolytic enzymes. As a result, NF- $\kappa$ B is activated and translocated into the nucleus. I $\kappa$ B $\alpha$  was analyzed by Western blot in order to identify the activation of NF- $\kappa$ B in the cytosol of A549 cells. Both IL-1 $\beta$  (Fig. 1A) and TNF- $\alpha$  (Fig. 1B) cause a rapid fall in the levels of I $\kappa$ B $\alpha$  within 2 h of exposure. The level of I $\kappa$ B $\alpha$  returns to normal within 4 h postexposure and remains unchanged through 24 h. In contrast, IFN- $\gamma$  did not cause a decrease in I $\kappa$ B $\alpha$  (Fig. 2C). When A549 cells are exposed to both IL-1 and TNF- $\alpha$  in combination (Fig. 2D) there is a synergistic (measured concentration different than predicted) decrease in I $\kappa$ B $\alpha$  within 2 h, followed by a synergistic increase in I $\kappa$ B $\alpha$  at 4 h to a higher level than predicted. This “rebound” or synergy effect is attenuated over the next 20 h (Fig. 1). Interestingly, IL-1 $\beta$ -induced I $\kappa$ B $\alpha$  degradation (or NF- $\kappa$ B activation) was augmented by IFN- $\gamma$  (see Fig. 1E at time = 2 h), whereas TNF- $\alpha$ -induced I $\kappa$ B $\alpha$  degradation was not effected (Fig. 2F). When all three cytokines are present (Fig. 2G), there is a mild synergistic effect at 24 h only.

**NF- $\kappa$ B translocation into the nucleus.** NF- $\kappa$ B is retained in the cytosol prior to cytokine exposure.



NF- $\kappa$ B occurs as a homodimer (p50/p50) or as a heterodimer (p65/p50). The cytosolic levels of p65 do not change appreciably following exposure to IL-1 $\beta$  (Figs. 2A and 2C, upper panels), whereas cytosolic p65 tends to increase following exposure to TNF- $\alpha$  (Fig. 2B, upper panel) over 24 h. In contrast, nuclear levels of NF- $\kappa$ B are increased dramatically above baseline within 2 h of exposure to either TNF- $\alpha$  or IL-1 $\beta$  (Figs. 2A and 2B, lower panels), but are unchanged following exposure to IFN- $\gamma$  (Fig. 2C, lower panel). When the cells were exposed to both IL-1 $\beta$  and TNF- $\alpha$ , the nuclear response is exaggerated (near additive), but is not synergistic (Fig. 2D, lower panel). These results are consistent with Fig. 1.

*iNOS expression following exposure of cytokine(s).* All three cytokines can induce mild (1–2.5% of maximal response) iNOS monomer synthesis within 12 h of exposure (Figs. 3A–3C). The levels of iNOS peak 24–48 h following exposure. The interaction between IL-1 $\beta$  in combination with TNF- $\alpha$  and/or IFN- $\gamma$  is strongly synergistic (Figs. 3D and 3E), particularly that between IL-1 $\beta$  and IFN- $\gamma$  (~55% of maximal response); however, the synergy between TNF- $\alpha$  and IFN- $\gamma$  (Fig. 3F) is mild (increase from 2.5 to 5% of maximum). There may also exist a three-way synergy (Fig. 3G) between IL-1 $\beta$ , TNF- $\alpha$ , and IFN- $\gamma$ , particularly at 24 and 72 h. This pattern of iNOS expression following exposure of cytokines is consistent with that seen for activation and translocation of NF- $\kappa$ B with the notable exception that IFN- $\gamma$  can induce iNOS expression independently, yet does not activate NF- $\kappa$ B.

*Effects of BH4, FAD, FMN, and L-arginine on NO production.* Three cofactors (FAD, FMN, and BH4) and L-arginine play important roles in iNOS dimerization and its catalytic activity. Exogenous cofactors were added to the media of A549 monolayers in order to investigate their effects on NO production. All three cofactors can affect NO production in the

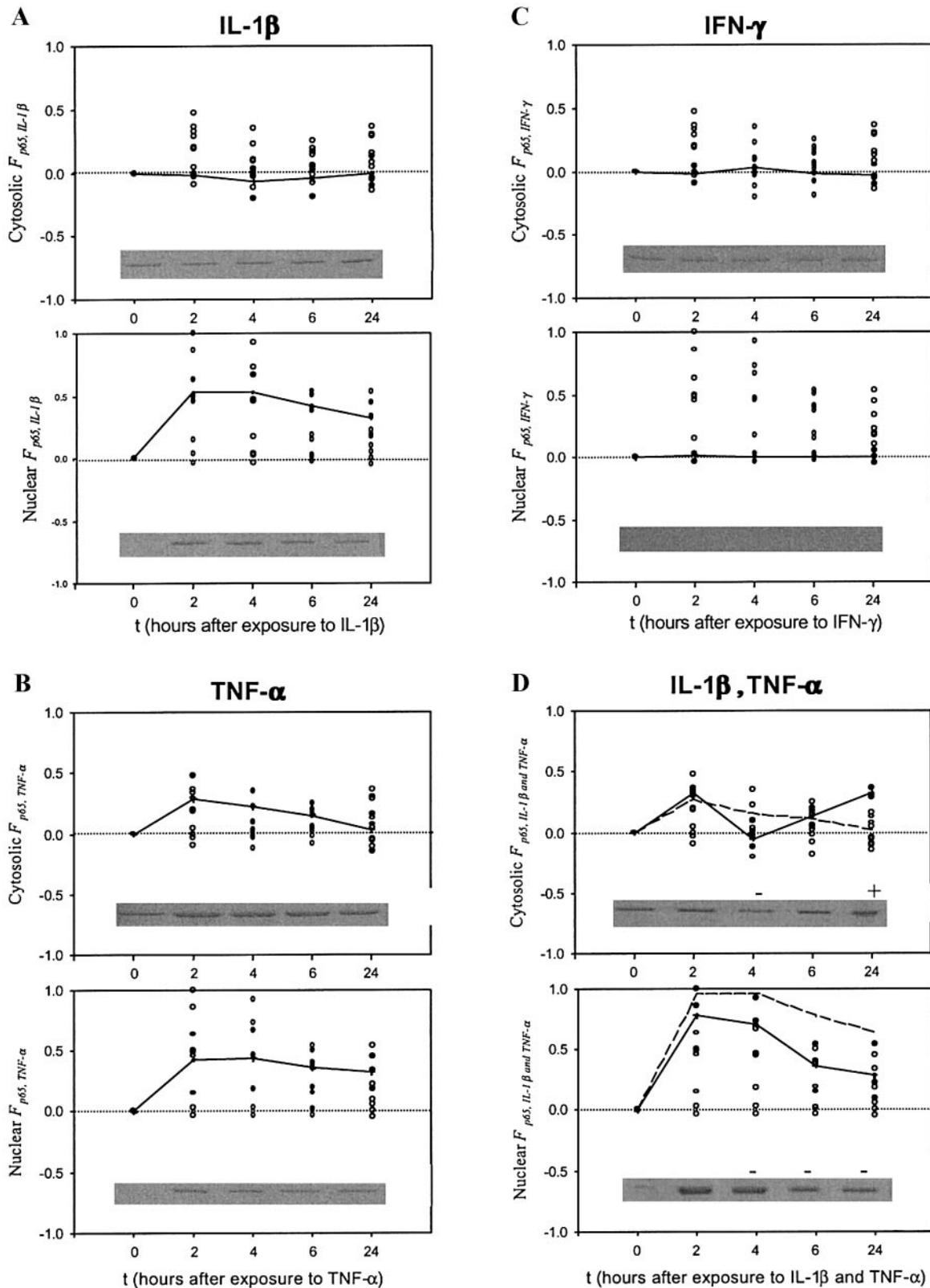
absence of cytokines. We determined (data not shown) the optimal concentrations of each cofactor and L-Arginine independently to produce a maximum production of NO in the absence of cytokines. The optimal concentration was defined as the concentration necessary to achieve 90% of the maximal response. The following optimal concentrations for FAD, FMN, BH4, and L-arginine, respectively, were found: 10  $\mu$ M, 5  $\mu$ M, 0.1  $\mu$ M, and 2 mM. These concentrations were then utilized to determine the relationship between the cytokines and the cofactors and L-Arginine. FAD, FMN, and L-Arginine did not affect *cytokine-induced* NO production appreciably (data not shown). However, exogenous BH4 did impact *cytokine-induced* NO production (Fig. 4).

To determine whether additional exogenous BH4 can augment cytokine-induced NO production, Fig. 4 presents the change in NO concentration following addition of 0.1  $\mu$ M BH4 relative to the NO concentration in the presence of the cytokine or cytokine combination in the absence of BH4. Exogenous BH4 (0.1  $\mu$ M) enhanced NO production in the presence of individual cytokines, but only the augmentation in the presence of TNF- $\alpha$  was larger than that induced by BH4 in the absence of cytokines. NO production in response to *combinations* of cytokines was not enhanced by exogenous BH4 with the notable exception of the combination of TNF- $\alpha$  and IFN- $\gamma$  (Fig. 4).

## DISCUSSION

The goal of this study is to provide evidence to support a possible mechanism(s) of synergistic cytokine-induced NO production in human alveolar epithelial cells. Our previous study documented two-way or three-way synergistic interaction among IL-1 $\beta$ , TNF- $\alpha$ , and IFN- $\gamma$  in NO production in human alveolar epithelial cells (A549) (12). The current

**FIG. 1.** Changes of I $\kappa$ B $\alpha$  in the presence of the following combinations of cytokines: (A) IL-1 $\beta$ , (B) TNF- $\alpha$ , (C) IFN- $\gamma$ , (D) IL-1 $\beta$  and TNF- $\alpha$ , (E) IL-1 $\beta$  and IFN- $\gamma$ , (F) TNF- $\alpha$  and IFN- $\gamma$ , and (G) IL-1 $\beta$ , TNF- $\alpha$ , and IFN- $\gamma$ . I $\kappa$ B $\alpha$  was analyzed by Western blot at 2-, 4-, 6-, 24-h interval. The solid circles (●) represent two experiments for a specific cytokine or cytokine combination (i.e., IL-1 $\beta$  for A). The solid lines with small cross (+) represent the average of the two experiments (●). Dashed lines represent predicted values calculated by Eqs. [3] or [5]. The open circles (○) are the data from all other cytokine combinations (i.e., for A, all data except that for IL-1 $\beta$ ) for comparative purposes. +: statistically significant positive difference between predicted and observed values; -: statistically significant negative difference between predicted and observed values.



study explores several molecular steps in the NO synthesis pathway as potential points of synergistic action.

**Transcription factor NF- $\kappa$ B and I $\kappa$ B $\alpha$ .** IL-1 $\beta$  and TNF- $\alpha$  have previously been shown to activate NF- $\kappa$ B as an initial step in their mechanism of inducing NO synthesis (5, 21). In contrast, IFN- $\gamma$  induces NO production through a different transcription factor, IRF-1 (22). Certainly one possible mechanism of synergy between two cytokines is enhanced activation and translocation of NF- $\kappa$ B from the cytosol to the nucleus. Marks-Konczalik and coworkers indicated that NF- $\kappa$ B binding efficiency was strongly correlated with activation and translocation of NF- $\kappa$ B in A549 cells (22). IL-1 $\beta$ - and TNF- $\alpha$ -induced activation/translocation of NF- $\kappa$ B was not synergistic, but IFN- $\gamma$  did cause a synergistic activation of IL-1 $\beta$ -induced activation/translocation of NF- $\kappa$ B. This potential source of synergy (additional free NF- $\kappa$ B available to translocate to nucleus) is shown schematically in Fig. 5.

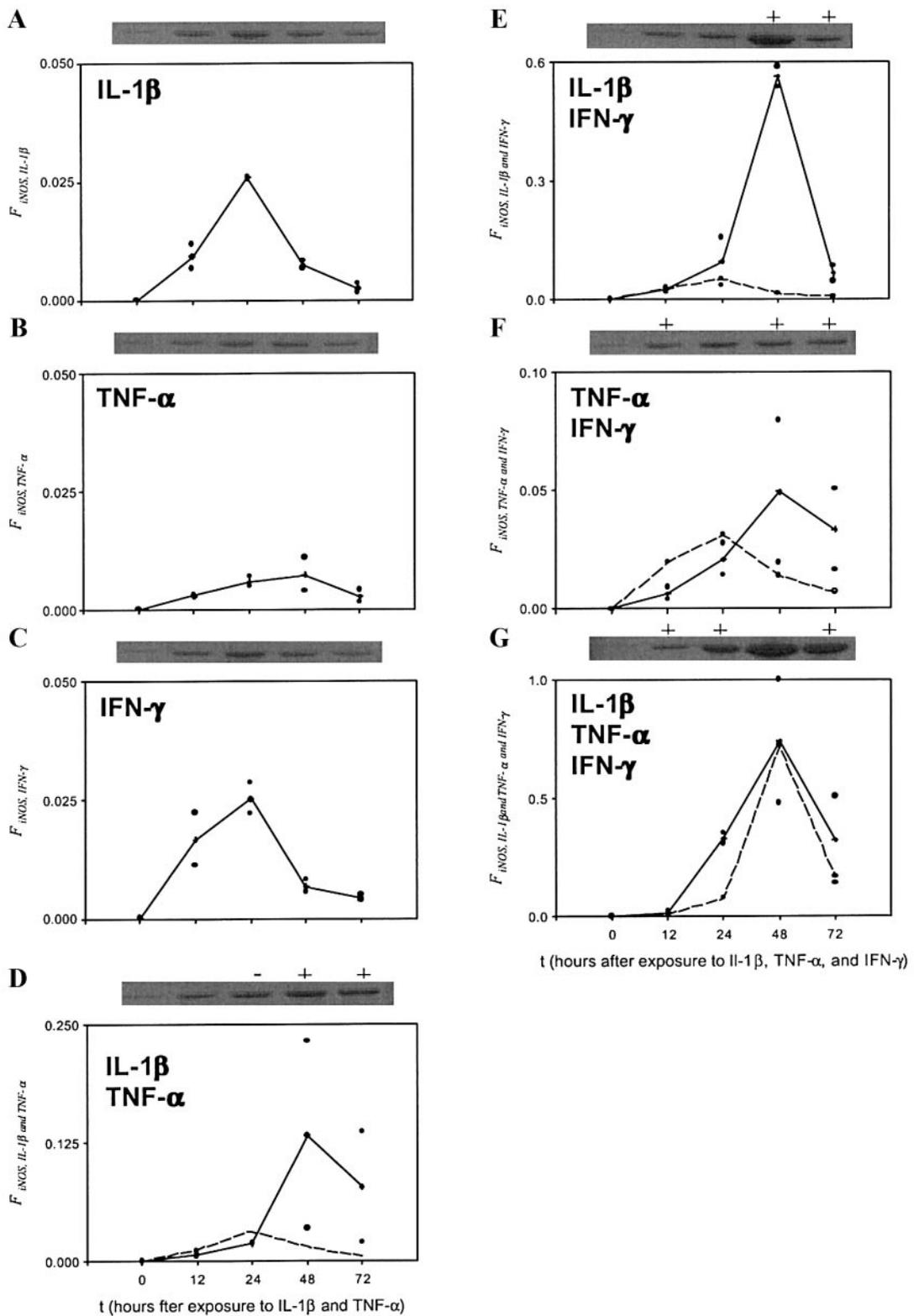
Reappearance of I $\kappa$ B $\alpha$  four hours following exposure (Fig. 1) correlates with the termination of the activation of NF- $\kappa$ B (15). Four hours following exposure, the levels of I $\kappa$ B $\alpha$  returns to normal or higher levels, probably due to new protein synthesis (15). After the degradation of the initial amount of I $\kappa$ B $\alpha$ , cytosolic I $\kappa$ B $\alpha$  is newly formed by an accelerated production of the protein. This new protein production is, at least in part, transcriptionally regulated (23) by the interaction of NF- $\kappa$ B with DNA-binding sites located in the promoter of the I $\kappa$ B $\alpha$  gene (24, 25). Recent researchers hypothesized that large amounts of newly synthesized I $\kappa$ B $\alpha$  could exceed the capacity of cytosolic NF- $\kappa$ B proteins to associate with the inhibitor, thus permitting free I $\kappa$ B $\alpha$  to accumulate in the nucleus (26, 27). The "rebound" effect of I $\kappa$ B $\alpha$  observed for time >6 h following exposure in Fig. 1 was higher in the presence of IL-1 $\beta$  with *either* TNF- $\alpha$  or IFN- $\gamma$  and was much higher

among all three cytokines. It is unlikely that reaccumulation of I $\kappa$ B $\alpha$  is directly related to synergistic cytokine-induced iNOS expression (Fig. 3) and NO production (12), but may play a role in the cell's ability to respond to consecutive inflammatory stimuli.

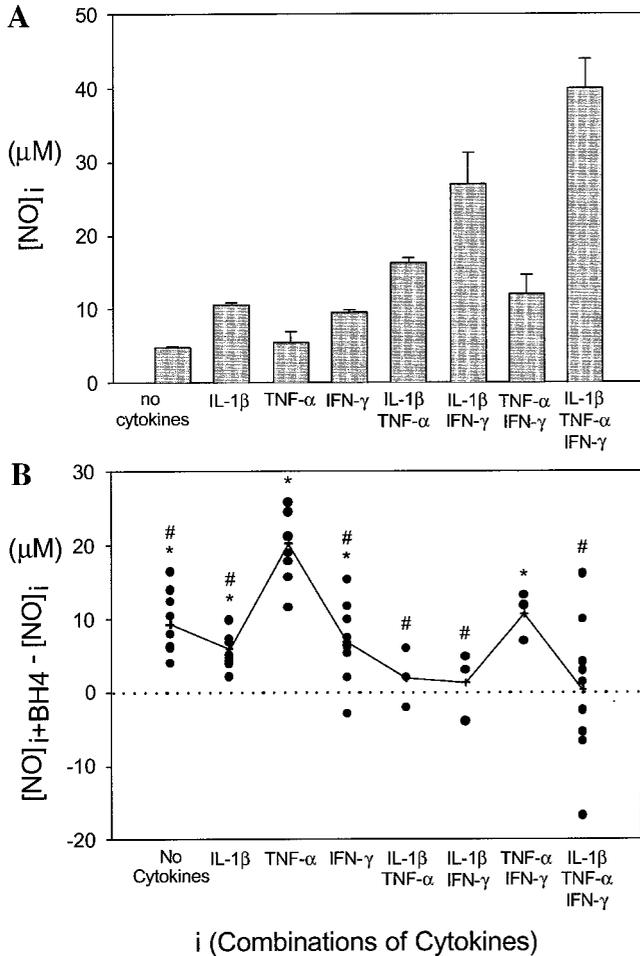
Although the *amount* of nuclear NF- $\kappa$ B observed in the presence of both IL-1 $\beta$  and TNF- $\alpha$  is not synergistic (closer to additive), this does not rule out this as a possible mechanism of synergy. An increased rate of translocation of NF- $\kappa$ B and/or the cotranslocation of other transcription factors results in an increased rate of transcriptional initiation and elongation. Through the direct functional synergistic interaction between transcription factors in promoter activity, both DNA binding affinity and complex stability can be enhanced, resulting in a highly stable multiprotein complex (5). The stable multiprotein complex may affect both posttranscriptional and posttranslational stability. In other words, we cannot rule out the possibility that an increased amount of nuclear NF- $\kappa$ B (not a synergistic amount) may have a nonlinear (or synergistic) effect on both posttranscriptional and posttranslational stability and result in synergistic concentrations of iNOS monomers (Fig. 5).

**iNOS expression.** Our data demonstrate synergistic interaction between IL-1 $\beta$  in combination with IFN- $\gamma$  and/or TNF- $\alpha$  on iNOS expression. In the presence of all three cytokines, there is not a convincing three-way synergy. Synergy between TNF- $\alpha$  and IFN- $\gamma$  is subtle and cannot be ruled out, particularly 72 h following exposure (note the difference in scale of Figs. 4E and 4F). This pattern of synergy is consistent with the discussion above regarding the presence of additional transcription factors (Fig. 5). These findings provide strong evidence that the observed synergy in NO-production may be tightly linked to the amount of iNOS expressed. Recall, that TNF- $\alpha$  and IFN- $\gamma$  do not exhibit synergy in A549 NO

**FIG. 2.** Nuclear (lower panel) and cytosolic (upper panel) distribution of NF- $\kappa$ B in the presence of the following combinations of cytokines: (A) IL-1 $\beta$ , (B) TNF- $\alpha$ , (C) IFN- $\gamma$ , (D) IL-1 $\beta$  and TNF- $\alpha$ . (a) cytosolic NF- $\kappa$ B, (b) nuclear NF- $\kappa$ B. NF- $\kappa$ B was analyzed by Western blot at 2-, 4-, 6-, 24-hour interval. The solid circles (●) represent three experiments for a specific cytokine or cytokine combination (i.e., IL-1 $\beta$  for A). The solid lines with small cross (+) represent the average of the two experiments (●). Dashed lines represent predicted values calculated by Eqs. [3] or [5]. The open circles (○) are the data from all other cytokine combinations (i.e., for A, all data except that for IL-1 $\beta$ ) for comparative purposes. +: statistically significant positive difference between predicted and observed values; -: statistically significant negative difference between predicted and observed values.



**FIG. 3.** Expression of iNOS protein monomer in the presence of the following combinations of cytokines: (A) IL-1 $\beta$ , (B) TNF- $\alpha$ , (C) IFN- $\gamma$ , (D) IL-1 $\beta$  and TNF- $\alpha$ , (E) IL-1 $\beta$  and IFN- $\gamma$ , (F) TNF- $\alpha$  and IFN- $\gamma$ , and (G) IL-1 $\beta$ , TNF- $\alpha$ , and IFN- $\gamma$ . INOS protein monomer was analyzed by Western blot at 12-, 24-, 48-, 72-h interval. The solid circles (●) represent two experiments for a specific cytokine or cytokine combination (i.e., IL-1 $\beta$  for A). The solid lines with small cross (+) represent the average of the two experiments (●). Dashed lines represent predicted values calculated by Eqs. [3] or [5]. The open circles (○) are the data from all other cytokine combinations (i.e., for A, all data except that for IL-1 $\beta$ ) for comparative purposes. +: statistically significant positive difference between predicted and observed values; -: statistically significant negative difference between predicted and observed values.



**FIG. 4.** (A) Baseline cytokine-induced nitric oxide production. (B) Effect of exogenous BH4 (0.1 μM) on cytokine-induced NO production relative to the baseline in (A). NO was measured at 96 h following exposure of cytokine(s). \*Statistically different from baseline. #Statistically different from TNF-α alone.

production (12), and the observed synergy in iNOS monomer expression is minimal relative to that between IL-1β and TNF-α and between IL-1β and IFN-γ.

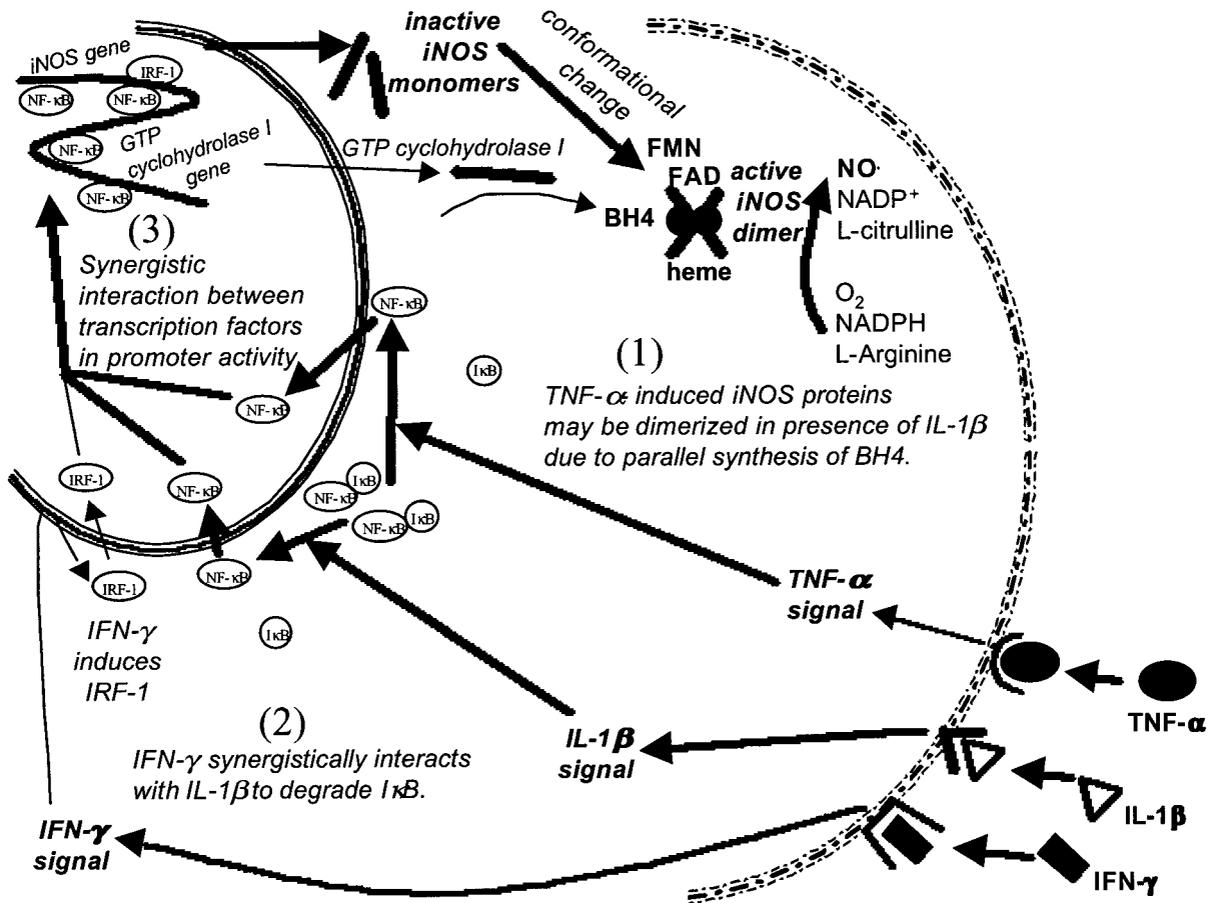
Although human iNOS is regulated primarily at the level of transcription, we have not analyzed our cultures for iNOS mRNA. Instead, we chose to focus on the functional protein and its dimerization. It may be difficult to quantitatively compare the results of Northern Blot (mRNA) with Western, due to low mRNA stability. Mellott *et al.* (28) recently demonstrated that iNOS mRNA was not induced by a single cytokine in A549 cells. Although iNOS protein

was detected, iNOS mRNA could not be detected. This could be due to the relative instability of mRNA, as combinations of cytokines may enhance iNOS mRNA stability (29). However, this discrepancy may also be due to differences in the protocol. Our protocol utilized higher cytokine concentrations (100 ng/ml) which we had previously determined produced the maximum NO production (12). In addition, cytokine-induced iNOS mRNA can be inhibited by residual serum in culture media (9). In order to minimize this inhibition, cells in the current study were preincubated in serum-free media for 24 h, prior to cytokine exposure for 24 h. This contrasted with the study of Mellott *et al.* (28), which used serum-free media for 8 hours prior to cytokine exposure for 8 h.

A particularly interesting result is the difference between the interaction of IFN-γ with IL-1β or TNF-α. Recall, that both IL-1β and TNF-α induce iNOS monomer synthesis through activation of NF-κB, yet IFN-γ is able to cause a dramatically larger synergistic increase in iNOS monomer synthesis when paired with IL-1β rather than TNF-α. This suggests distinctly different signaling cascades between IL-1β and TNF-α. These differences might be explained, in part, by the interaction of the cytokines with cofactors for iNOS dimerization and activation.

*Cofactors for iNOS dimerization.* There are three cofactors necessary to produce an active iNOS dimer: FMN, FAD, and BH4. It is certainly reasonable to presume the synthesis of these co-factors parallels the synthesis of the iNOS monomers, and there is evidence that Guanosine 5'-triphosphate (GTP) cyclohydrolase I, the rate limiting enzyme of BH4 biosynthesis, is induced in parallel to NOS induction (30) (see Fig. 5). This provides a potential point for synergy amongst the cytokines. Our data demonstrate that TNF-α induced NO production can be significantly enhanced by additional BH4, suggesting that inadequate amounts of BH4 are available upon exposure to TNF-α in A549 cells relative to that available when the cells are exposed to IL-1β and INF-γ.

NOS dimers have two heme groups that are essential for catalysis. BH4 stabilizes the dimeric, active state of the enzyme. The availability of the heme appears to be the most critical parameter for



**FIG. 5.** Plausible two-way or three-way synergistic interaction among three cytokines in NO production in human alveolar epithelial cells (A549). Solid arrows represent known pathways based on evidence in the literature (thin) or that presented in this study (bold face). (1) The synergy in NO production observed in the presence of IL-1 $\beta$  and TNF- $\alpha$  may be due to parallel BH4 synthesis in the presence of both cytokines. (2) IFN- $\gamma$  synergistically interacts with IL-1 $\beta$  to enhance the degradation of I $\kappa$ B. (3) Direct functional synergistic interaction in promoter activity between transcription factors in the presence of IL-1 $\beta$  and IFN- $\gamma$ .

correct assembly of the active dimer, and formation of a high-affinity binding site for BH4 (30). Interestingly, NO, the final product, inhibits the binding of transcription factors to its cognate sequences on iNOS DNA and can also attenuate the process of dimerization, conformational change, and activation, by forming a heme-NO complex (31). BH4 indirectly scavenges free NO by reaction with superoxide that is formed in the course of autooxidation of BH4 (32). In addition, allosteric actions of BH4 on the iNOS domain destabilize heme-NO complex. Thus, newly synthesized iNOS monomers require an adequate supply of BH4 to become active, and to remain active.

Although TNF- $\alpha$  can induce the synthesis of iNOS monomers, it cannot induce NO production alone in A549. This could be due to inadequate concentrations of BH4. The synergy in NO production observed in the presence of IL-1 $\beta$  and TNF- $\alpha$  may be due to parallel BH4 synthesis when IL-1 $\beta$  is present with one of the other cytokines (e.g., IL-1 $\beta$  and TNF- $\alpha$ ). Interestingly, the combination of IFN- $\gamma$  and TNF- $\alpha$  does not produce NO synergistically, and NO production in the presence of exogenous BH4 is enhanced. This suggests that in the presence of both IFN- $\gamma$  and TNF- $\alpha$ , perhaps IFN- $\gamma$  can induce cellular synthesis of BH4, but not in adequate amounts to capitalize on the additional iNOS monomers present from TNF- $\alpha$ .

**Conclusions.** The observed synergy in NO production between IL-1 $\beta$ , IFN- $\gamma$ , and TNF- $\alpha$  has complex underlying mechanisms. We conclude that the synergistic response in A549 cells is due, in part, to three mechanisms: (1) synergy in iNOS monomer synthesis, (2) synergy in NF- $\kappa$ B activation, and (3) synergy in parallel BH4 synthesis. The increased level of iNOS synthesis is likely the result of non-linear interactions between several transcription factors including NF- $\kappa$ B and IRF-1. BH4 is an essential cofactor synthesized in parallel with the expression of inflammatory proteins, and whose intracellular level appears to be regulated by cytokines. Future work must continue to elucidate these mechanisms if inflammatory induced NO production can be fully characterized and thus manipulated for therapeutic potential.

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