NITRIC OXIDE: Biology and Chemistry
Vol. 3, No. 4, pp. 348–357 (1999)
Article ID niox.1999.0242, available online at http://www.idealibrary.com on IDEAL®

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

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Received April 2, 1999, and in revised form May 24, 1999

Nitric oxide (NO) is an important mediator molecule in regulating normal airway function, as well as in the pathophysiology of inflammatory airway diseases. In addition, cytokines are potent messenger molecules at sites of inflammation. The specific relationship among IL-1β, TNF-α, and IFN-γ on iNOS induction and NO synthesis in *human* alveolar epithelial cells has not been determined. In addition, rigorous methods to determine potential synergistic action between the cytokines have not been employed. We exposed monolayer cultures of A549 cells to a factorial combination of three cytokines (IL-1β, TNF-α, and IFN-γ) and three concentrations (0, 5, and 100 ng/mL). TNF-α alone does not induce NO production directly; however, it does have a stimulatory effect on IL-1β-induced NO production. IL-1β and INF-γ both induce NO production alone, yet at different concentration thresholds, and act synergistically when present together. In the presence of all three cytokines, the net effect of NO production exceeds the predicted additive effect of each individual cytokine and the two-way interactions. Several plausible mechanisms of synergy among IL-1β, TNF-α, and IFN-γ in NO production from human alveolar epithelial cells (A549) are proposed. In order to verify the proposed mechanisms of synergy, future experimental and theoretical studies must address several molecular steps through which the iNOS gene is expressed and regulated, as well as the expression and regulation of enzyme cofactors and substrates.

Key Words: **synergy; A549; interleukin-1β; interferon-γ; tumor necrosis factor-α.**

Nitric oxide (NO) is a highly reactive and pervasive biological mediator produced by mammalian cells, and its physiological actions are broad. In general, NO-mediated functions fall into three categories: (1) smooth muscle relaxation (1, 2), (2) neurotransmission (3), and (3) cell-mediated immune response (4, 5). The immune response of the lungs is complex, and there is growing evidence that NO plays an important role. Recently, investigators have demonstrated the potential for human lung epithelial cells to express inducible nitric oxide synthase (iNOS) and produce NO (6–8).

iNOS gene expression is regulated by complex mechanisms. Agents that induce iNOS expression include various cytokines (IL-1β, TNF-α, and IFN-γ), endotoxin (lipopolysaccharide, LPS), and a host of other agents (9). Cytokines are regulatory polypeptides produced and secreted by a wide variety of cell types. These polypeptides include the interleukins, tumor necrosis factors, interferons, and others. In the lung and respiratory tract, cytokines are involved in the infammatory response, and ultimately with the development of disease (10). Hence, it is not surprising that a relationship exists among NO production in lung cells and cytokines.

In 1994, Robbins *et al.* (7) and Asano *et al.* (6) both

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demonstrated that a human Type II alveolar epithelial cell line (A549) could express iNOS and produce NO in response to a combination of IL-1β, TNF-α, and IFN-γ. However, these studies did not investigate the potential effect of cytokine concentration on the observed synergy, and did not include a rigorous statistical model to determine specific one-way and even two-way interactions. Gutierrez *et al.* (11) proposed that combinations of stimuli are often required to induce the *in vitro* production of NO. Hence, they exposed rat fetal Type II alveolar cells to different combinations of IL-1β, TNF-α, and IFN-γ and demonstrated that all three cytokines present together induced the largest production of NO, but that TNF-α and IFN-γ alone did not significantly affect NO synthesis. iNOS induction by cytokines has been shown to be species specific (12); the specific relationship between IL-1β, TNF-α, and IFN-γ on iNOS induction and NO synthesis in *human* alveolar cells has not been determined. We hypothesized that human alveolar epithelial cells (A549) would respond in a similar fashion to combinations of cytokines as the rat alveolar cells. The goal of this current study is to determine the specific synergistic relationship between IL-1β, TNF-α, and IFN-γ in a human alveolar type II cell line and propose plausible mechanisms for the observed synergy in NO production.

MATERIALS AND METHODS

Materials

Dulbecco's modified eagle's medium (DMEM), Dulbecco's phosphate-buffered saline, Trypsin–EDTA solution, antibiotics (penicillin–streptomycin, amphotericin B), recombinant human interleukin-1β (IL-1β), recombinant human tumor necrosis factor-α (TNF-α), and recombinant human interferon-γ (IFN-γ) 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).

Cell Culture

Immortal Type II alveolar cell lines of human origin (A549) were obtained from the American Type Culture Collection. A549 is epithelial-like in morphology and originates from a human lung carcinoma. The cells were seeded at 4×10^4 cells/cm^2 onto 48-well plates (Corning Costar) containing 0.5 ml of DMEM supplemented with 10% FBS, L-glutamine (2 mM), amphoterincin B (5.6 mg/L), and penicillin–streptomycin (100 U/mL) and allowed to grow to confluency.

Exposure to Cytokines

Following confluency, the cells were incubated in serum-free media (DMEM) for 24 h prior to cytokine exposure. Baseline concentration of NO in the media due to constitutive NOS (cNOS) was determined initially. Monolayers were initially exposed to a combination of cytokines ranging in concentration from 1 to 100 ng/ml for a period of time up to 120 h to determine the point of maximum NO production. Once this was determined, the monolayers were then exposed to a factorial combination of three cytokines (IL-1β, TNF-α, and IFN-γ) at three concentrations (0, 5, and 100 ng/mL) with or without the presence of 0.1 mM L-N^G arginine methyl ester (L-NAME). L-NAME is a competitive inhibitor of NOS and can be used to determine the source of NO synthesis. Stable end-products of NO oxidation (NO_2^- and NO_3^-) were then measured using chemiluminescence (Model 280 NOA, Sievers, Inc., Boulder, CO) in the media bathing the apical surface of the cells at 24-h intervals for 96 h following exposure. The number of viable cells at each time interval was measured by trypan blue dye-exclusion. Cell mass was determined by a modification of the colorimetric Lowry assay for total cell protein, using a phenol reagent (Folin–Ciocalteau) for the development of color (13). Each exposure was repeated in duplicate, and NO concentration was normalized by the cell mass in each well.

Nitric Oxide Assay

NO activity was analyzed by chemiluminescence (Model 280 NOA, Sievers, Inc., Boulder, CO). NO is highly unstable in the presence of oxygen, and is rapidly converted into NO_2^- and NO_3^- in liquid media. In order to detect NO, both NO_2^- and NO_3^- 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

To determine two-way (interaction between two cytokines) synergy among the cytokines in NO production, observed concentration of NO (C^{O}_{NO}) was compared with the predicted concentration of NO ($C^{P,II}_{NO}$). $C^{P,II}_{NO}$ was calculated assuming no interaction or synergy among the cytokines by the equations

$$C^{P,II}_{NO} = C^{i}_{NO,X} + C^{i}_{NO,Y} + C^{C}_{NO} \quad [1a]$$

$$C^{i}_{NO,X\text{ or }Y} = C^{O}_{NO,X\text{ or }Y} - C^{C}_{NO}, \quad [1b]$$

where $C^{i}_{NO,XorY}$ is the concentration of NO induced by cytokine X or Y (X or Y equals IL-1β, TNF-α, or IFN-γ) from iNOS. The superscripts "O", "P", "i", and "C" refer to observed, predicted, induced, and constitutive concentrations of nitric oxide, respectively.

To determine whether a three-way (interaction among three cytokines) synergy between the cytokines exists, the predicted concentration must account for the observed individual inductions as well as two-way interactions. The three-way predicted concentration, $C^{P,III}_{NO}$, is then given by the equations

$$C^{P,III}_{NO} = C^{i}_{NO,IL} + C^{i}_{NO,IFN} + C^{i}_{NO,TNF} + C^{i}_{NO,IL-TNF} + C^{i}_{NO,IL-IFN} + C^{i}_{NO,IFN-TNF} + C^{C}_{NO} \quad [2a]$$

$$C^{i}_{NO,IL-TNF} = C^{O}_{NO,IL-TNF} - C^{i}_{NO,IL} - C^{i}_{NO,TNF} - C^{C}_{NO}$$

$$C^{i}_{NO,IL-IFN} = C^{O}_{NO,IL-IFN} - C^{i}_{NO,IL} - C^{i}_{NO,IFN} - C^{C}_{NO}$$

$$C^{i}_{NO,TNF-IFN} = C^{O}_{NO,TNF-IFN} - C^{i}_{NO,TNF} - C^{i}_{NO,IFN} - C^{C}_{NO}, \quad [2b]$$

where $C^{i}_{NO,X-Y}$ (X or Y equal to IL-1β, TNF-α, or IFN-γ) is the concentration of NO induced by the possible two-way interaction of cytokine X and Y. For example, $C^{i}_{NO,IL-IFN}$ is the concentration of NO induced by the possible two-way interaction of IL-1β and IFN-γ from iNOS.

Statistical Analysis

There were three concentration levels for each of the three different treatments (i.e., cytokines), making a total of 27 different combinations of treatments. Each combination of treatments is referred to as a cell. As each experiment was performed twice, there were two measurements for each cell. The statistical model considered was a 3^3 factorial design. The linear model associated with this design incorporated all two-way and three-way interactions. The *F*-test rejected the null hypothesis of zero interaction. This reduced our analysis to performing pair-wise tests among the 27 cells using Fisher's least significant difference method. Because of the large number of pair-wise comparisons (351 to be precise) for the observed data, we did not decide on a pair-wise difference unless the *P*-value was very small ($P < 0.001$ for statistical significance). However, the more traditional critical *P*-value of 0.05 was used when comparing observed values to predicted values.

RESULTS

Cell Viability

NO concentrations (Fig. 1A) and cell viability (Fig. 1B) were simultaneously monitored at 24-h intervals for 120 h following exposure to cytokine mixtures to determine potential cytotoxicity of the exogenous cytokines or the increased concentrations of NO, NO_2^-, and NO_3^-. Cell viability decreased as time and concentrations of cytokine mixtures increased. When cytokine mixtures were added to the culture media, the number of viable cells, determined by trypan blue dye-exclusion assay, declined by more than 50% within 5 days at the maximal cytokine concentration (Fig. 1).

The viability of A549 cells was reduced more in the media containing TNF-α alone than IL-1β or IFN-γ alone (Fig. 2). The decrease in cell viability may be due to oxidative damage of the higher concentration of NO induced by cytokines. However, the half-life of the NO free radical is very short (order 1 s) and the NO free radical is rapidly changed into nitrite and nitrate. To test this hypothesis, A549 cells were exposed to exogenous nitrate (NO_3^-). As

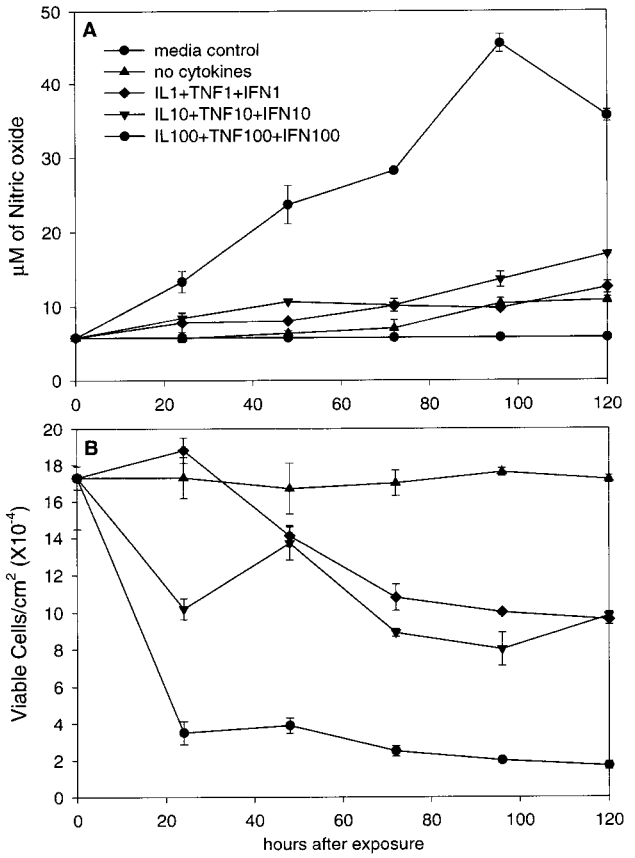

FIG. 1. Time profiles of nitric oxide production (A) and cell viability (B) for A549 monolayers treated with cytokine mixtures. Cell viability was determined by trypan blue dye-exclusion.

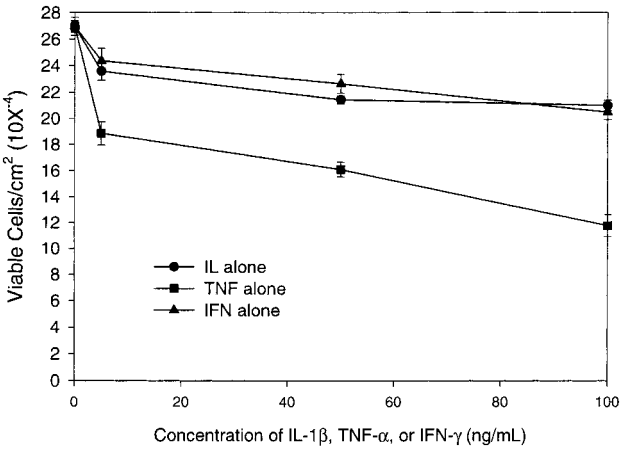

FIG. 2. Effects of IL-1β, TNF-α, or IFN-γ on cell viability. Viable cells were measured 96 h after exposure to each cytokine.

demonstrated in Fig. 3, exogenous nitrate (NO_3^-) in the absence of exogenous cytokines did not affect the cell viability and growth of A549 cells.

NO Production

Constitutive production. Nitric oxide was constitutively produced (cNOS) by A549 in the absence of exogenous cytokines at a basal level resulting in apical DMEM concentrations of 5.76–10.8 μM (Fig. 1). This constitutive production was completely eliminated with the addition of 0.1 mM L-NAME (data not shown). Cells exposed to cytokines (IL-1β, TNF-α, and IFN-γ) produced increased levels of nitric oxide (Fig. 1). Maximal dose-dependent response occurred 96 h after exposure using 100 ng/mL of each cytokine, and was approximately 500% larger than the basal level. Hence, the analysis of synergism between the cytokines was performed using the NO concentrations at 96 h and cytokine concentrations of 0, 5, or 100 ng/ml (Fig. 1).

Effect of individual cytokines. Figures 4B and 6A demonstrate that TNF-α alone did not significantly affect NO production in A549. In contrast, both IL-1β and IFN-γ significantly increased NO production from A549 over the basal or constitutive level. The effect of IFN-γ (Figs. 5B and 6B) is only observed at the largest concentration (100 ng/ml), whereas the effect of IL-1β is observed at the lowest

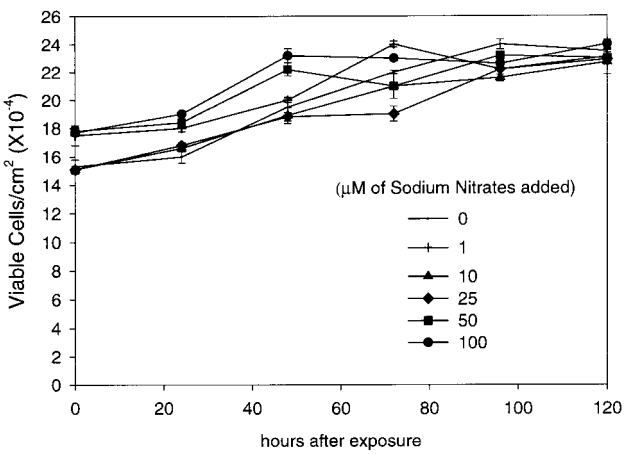

FIG. 3. Effects of exogenous nitrate (NO_3^-) on cell growth and viability. Various concentrations (0–100 μM) of sodium nitrates were added to media on A549 monolayers to determine effects of cytokine-induced increased NO production on cell viability.

(data not shown) and highest concentrations (Figs. 4A and 5A).

Interaction between TNF-α and IL-1β. TNF-α cannot directly induce NO production (Figs. 4B and 6A), but it has a stimulatory or synergistic effect on IL-1β-induced NO production (Fig. 4). This stimulatory effect, as determined by the fact that $C_{NO}^{P,II}$ is less than the observed concentration, is observed at the higher concentration of TNF-α (100 ng/ml) and at both the high (100 ng/ml) and low (5 ng/ml) concentrations of IL-1β.

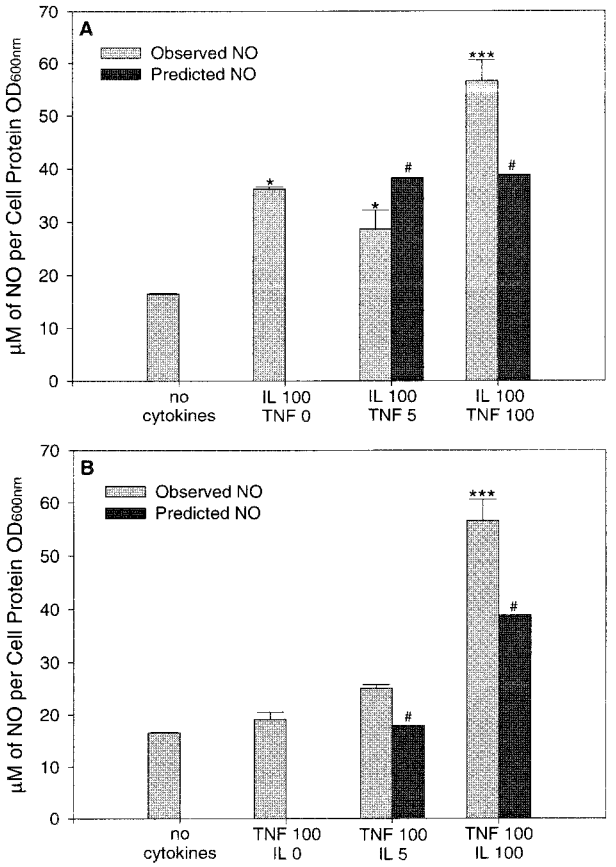

FIG. 4. Two-way interaction between IL-1β and TNF-α in NO production at 96 h after exposure to various concentrations of cytokine mixtures. (A) Concentrations of TNF-α were varied under high concentrations of IL-1β. (B) Concentrations of IL-1β were varied under high concentrations of TNF-α. Observed experimental concentrations are shown in light bars, and the predicted NO concentrations ($C_{NO}^{P,II}$, dark bars) are calculated based on Eq. 1. *Significantly higher than control (no cytokines), **Significantly higher than left two observed values, ***significantly higher than left three observed values, respectively ($P < 0.001$). #Significant difference compared with observed value ($P < 0.05$).

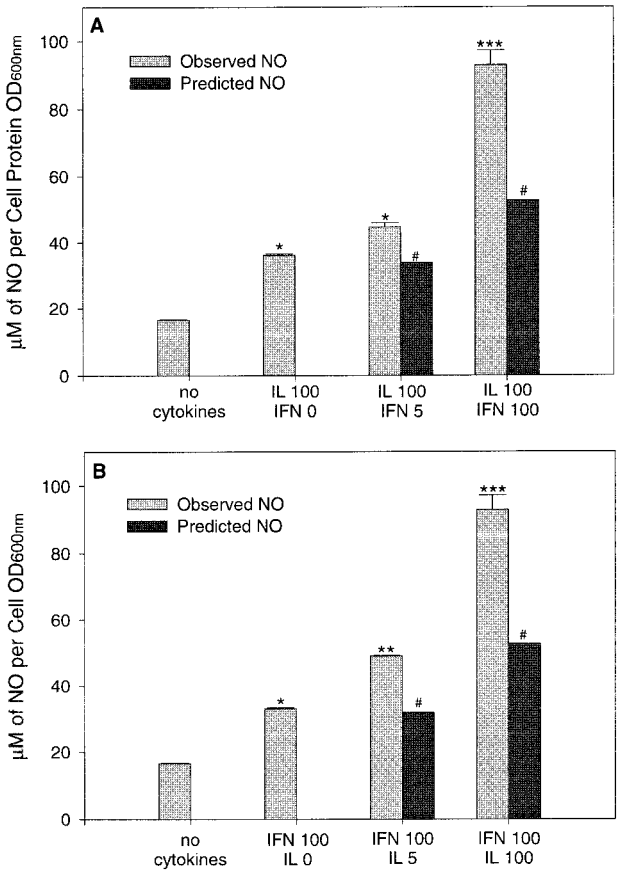

FIG. 5. Two-way interaction between IL-1β and IFN-γ in NO production at 96 h after exposure to various concentrations of cytokine mixtures. (A) Concentrations of IFN-γ were varied under high concentrations of IL-1β. (B) Concentrations of IL-1β were varied under high concentrations of IFN-γ. Observed experimental concentrations are shown in light bars, and the predicted NO concentrations ($C_{NO}^{P,II}$, dark bars) are calculated based on Eq. 1. *Significantly higher than control (no cytokines), **significantly higher than left two observed values, ***significantly higher than left three observed values, respectively ($P < 0.001$). #Significant difference compared with observed value ($P < 0.05$).

Interaction between IL-1β and IFN-γ. IFN-γ and IL-1β are both capable of inducing NO production alone, but together they produce more NO than predicted based on a simple sum of their individual effects (Fig. 5). This observed synergy is different from that of TNF-α on IL-1β-induced NO production in that the synergy is observed at both high and low concentrations (either 5 ng/ml or 100 ng/ml) of either IL-1β and IFN-γ.

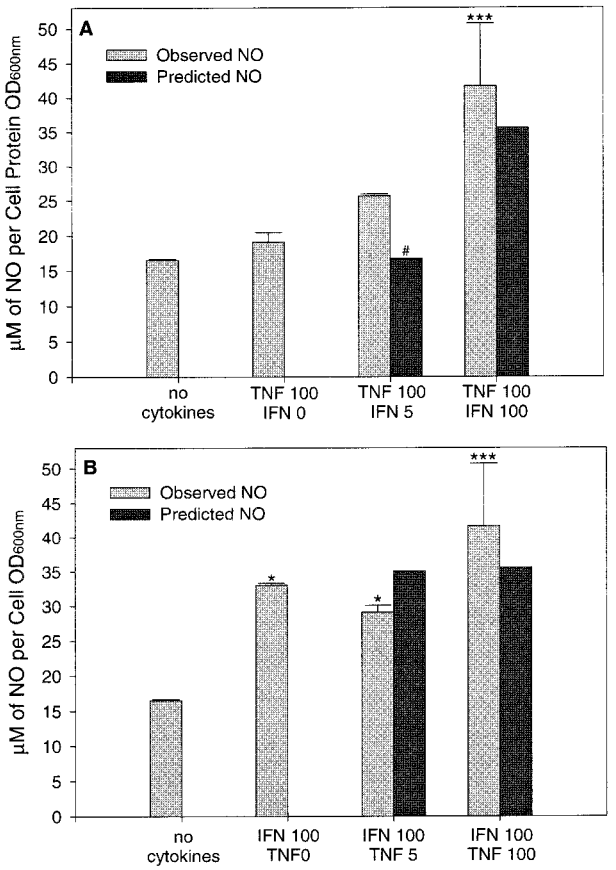

FIG. 6. Two-way interaction between TNF-α and IFN-γ in NO production at 96 h after exposure to various concentrations of cytokine mixtures. (A) Concentrations of IFN-γ were varied under high concentrations of TNF-α. (B) Concentrations of TNF-α were varied under high concentrations of IFN-γ. Observed experimental concentrations are shown in light bars, and the predicted NO concentrations ($C_{NO}^{P,II}$, dark bars) are calculated based on Eq. 1. *Significantly higher than control (no cytokines), **significantly higher than left two observed values, ***significantly higher than left three observed values, respectively ($P < 0.001$). #Significant difference compared with observed value ($P < 0.05$).

Interaction between TNF-α and IFN-γ. Although TNF-α can stimulate or enhance IL-1β-induced NO production, this is not observed on IFN-γ-induced NO production (Fig. 6). The combinations of TNF-α and IFN-γ produce NO at levels that are consistent with the separate actions of the individual cytokines. This suggests no interaction or synergism between TNF-α and IFN-γ in A549 NO production.

Interaction among IL-1β, TNF-α, and IFN-γ. Figure 7 demonstrates that NO production is higher than predicted in the presence of all three cytokines. When all three cytokines are present at 100 ng/ml, the media concentration of NO (138 μM) is larger than that predicted (119 μM) based on the actions of the individual cytokines and the two-way interactions described above between IL-1β and TNF-α, and between IL-1β and IFN-γ (Eq. 2). This represents a three-way synergistic effect between the cytokines.

DISCUSSION

Cell Viability

The decrease in cell viability observed following exposure to the cytokines may be due to oxidative damage of NO induced by cytokines. However, when A549 cells were exposed to exogenous NO_3^- the cell viability and growth were not affected (Fig. 3). Wink and co-workers have proposed that NO actually protects against cellular damage and cytotoxicity from reactive oxygen species (14) by reacting with them (i.e., superoxide anion) and acting as a savenger. Another possibility for the decrease in cell viability could be due to the toxicity of cytokines, specifically

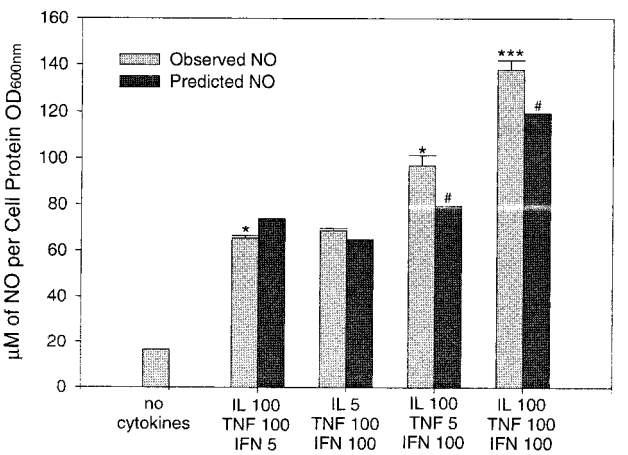

FIG. 7. Three-way interaction between IL-1β, TNF-α, and IFN-γ in NO production at 96 h after exposure to various concentrations of cytokine mixtures. Observed experimental concentrations are shown in light bars, and the predicted NO concentrations ($C_{NO}^{P,III}$, dark bars) are calculated based on Eq. 2. *Significantly higher than control (no cytokines), **significantly higher than left observed value as well as the control, respectively ($P < 0.001$). #Significant difference compared with observed value ($P < 0.05$).

TNF-α which demonstrated the greatest cytotoxicity. Carswell *et al.* demonstrated that TNF-α had selective toxicity for tumor cells and transformed cell lines (15). In contrast, Sugarman *et al.* showed an antiproliferative response of TNF-α in 16 of 34 tumor cell lines, while the growth of normal human cell lines from the colon, fetal skin, and lung was not inhibited (16). Our results indicate that it is plausible that the growth and proliferation of the A549 cell line is sensitive to TNF-α.

NO Production

Human alveolar epithelial cells (A549) have the capacity of releasing NO through the catalytic action of iNOS which is induced by cytokines. We have demonstrated in this study that specific combinations of cytokines stimulate the production of NO synergistically in a human alveolar epithelial cell line. Synergistic interaction between cytokines can occur when the individual cytokines impact different steps in the synthesis. Several plausible mechanisms of synergistic interactions can be proposed by our studies by considering the following steps in NO synthesis: transcription, posttranscription modification or processing, translation, and posttranslation.

The fundamental steps involved in cytokine-induced NO production either in human or animal cells is outlined in Fig. 8. Cytosolic transcription factors such as NF-κB and IRF-1 can diffuse into the nucleus and enhance the transcription of the iNOS gene to produce mRNA. The mRNA is then processed to produce the iNOS mRNA that can be translated to produce the inactive iNOS monomer protein. The inactive monomers then undergo a transmembrane conformational change that requires several cofactors such as FAD (flavin adenine dinucleotide), FMN (flavin mononucleotide), and BH4 (tetrahydrobiopterin) to produce the active dimer. The active dimer then converts L-arginine to L-citrulline in the presence of O_2 and NADPH and releases NO.

IL-1β, TNF-α, and IFN-γ are known inducers of iNOS. IL-1β-induced NO production in murine macrophages (17) and human A549 cells (18, 19) is transcriptionally regulated by NF-κB. Although NF-κB is activated in the cytosol by the removal of IκB, it is not clear if IL-1β increases the amount of free NF-κB, enhances the transport of free NF-κB from the cytosol to the nucleus, or enhances the binding of NF-κB to the promotor region of the iNOS gene. It is possible that IL-1β is involved in all three of these mechanisms at different concentration thresholds. Chu *et al.* (12) reported that IL-1β could not induce iNOS mRNA transcription at a concentration of 0.5 ng/ml in A549 cells. This concentration is an order of magnitude smaller than that used in our studies which demonstrated a significant increase in NO production. This certainly suggests a concentration threshold for the effect of IL-1β as an individual cytokine and cannot rule out effects of IL-1β that are not related to transcription.

IFN-γ-induced NO production has been shown to be regulated by another transcription factor, IRF-1, in murine macrophages (20). NF-κB, and a third transcription factor AP-1 (activator protein 1), are both involved when all three cytokines induce NO production in A549 cells (21). The probable mechanism of TNF-α in enhanced NO production is not well characterized, but it has been recently demonstrated that TNF-α alone can increase the binding of NF-kB to the promoter region of iNOS in A549 cells (18).

In addition, the effect of each cytokine on the induction of iNOS is both species and cell specific. For example, TNF-α alone can induce iNOS in both rat and mouse lung fibroblasts (22) whereas, rat type II alveolar cells do not respond to TNF-α alone (11). The promoter region of the iNOS gene in human A549 cells requires different activator proteins, such as AP-1, when compared to murine cells (12).

TNF-α alone cannot induce NO production directly (Figs. 4B and 6A). This finding is consistent with previous results in rat type II alveolar cells (11). However, TNF-α enhances NO production in the presence of IL-1β (Fig. 4). A possible mechanism for this action is that TNF-α may stimulate the induction of NF-κB precursor mRNA, resulting in an increase in the level of NF-κB–IκB complex (Fig. 8). This would augment IL-1β-induced NO production, but would not allow TNF-α to directly induce NO production alone.

In the case of NO production in the presence of both IL-1β and IFN-γ, the net effect exceeds the predicted additive effect of each cytokine (Fig. 7).

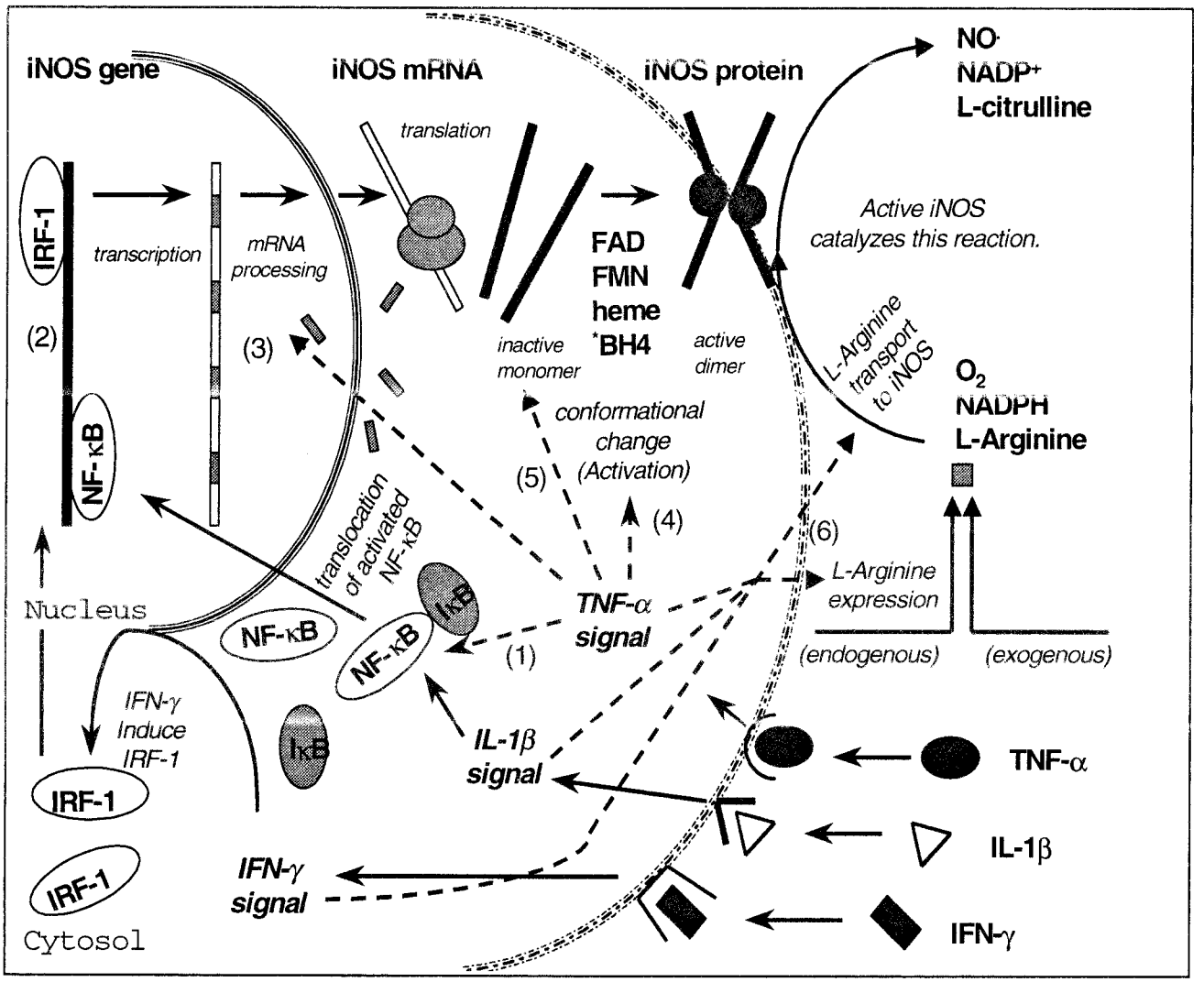

FIG. 8. Proposed mechanisms of two-way or three-way interaction among three cytokines. Solid lines represent known or probable pathways and mechanism based on evidence in the literature. Dashed lines represent hypotheses yet to be tested experimentally in human cells. NO is produced by the NOS enzyme, one of the largest (~300 kDa) and most complicated. NOS requires three cosubstrates (L-arginine, NADPH, and O_2) and five cofactors (FAD, FMN, calmodulin, tetrahydrobiopterin (BH_4), and heme). NO is produced by the catalytic action of NOS. The transcription of iNOS mRNA can be induced by cytokines. Nuclear factor-kappa B (NF-κB) and interferon regulating factor-1 (IRF-1) are involved in the transcriptional expression of iNOS gene (19, 20). Each cytokine cannot penetrate the cell membrane; rather, they attach to a transmembrane receptor and exert their effect through an intracellular signal. (1) Proposed mechanism of two-way interaction between IL-1β and TNF-α in NO production. TNF-α acts synergistically with IL-1β by increasing the level of the DNA–NF-κB complex. This could be due to increasing precursors of NF-κB. (2) Proposed mechanism of two-way interaction between IL-1β and IFN-γ in NO production. NF-κB and IRF-1 binding simultaneously to DNA may increase transcription efficiency/processing accuracy of iNOS mRNA. (3) TNF-α could inhibit the degradation of iNOS mRNA, or increase the transcription efficiency and processing accuracy of iNOS mRNA induced by IL-1β. (4) TNF-α may enhance the production of prosthetic groups (NADPH, FAD, FMN, and BH_4) that are required for the iNOS conformational change and its catalytic action for releasing NO. (5) TNF-α may inhibit the degradation of iNOS protein induced by IL-1β. (6) An inflammatory cytokine or cytokine mixture can upregulate substrate synthesis (L-arginine) and/or substrate uptake for the iNOS enzyme.

One possible mechanism for this synergy is that both NF-κB activated by IL-1β and IRF-1 induced by IFN-γ together enables iNOS DNA to transcribe iNOS mRNA more efficiently, compared to each cytokine alone (Fig. 8).

The mixture of TNF-α and IFN-γ produce NO at concentrations that are almost the same as the sum of the separate actions of the individual cytokines (Fig. 7). This suggests no interaction between TNF-α and IFN-γ. One possible hypothesis that is consistent with the above discussion is that TNF-α increases the synthesis of NF-kB (and hence the NF-κB–IκB complex) while IFN-γ increases the binding of IRF-1. Thus, increasing the NF-κB–IκB complex would not enhance IFN-γ-induced NO production.

The mixture of all three cytokines produces NO at concentrations that are larger than that predicted by the sum of the individual contributions and the two-way interactions (Fig. 7). Our previous discussion regarding possible mechanisms of actions of the cytokines might explain this observation. For example, if TNF-α increases the level of the NF-κB–IκB complex which is subsequently activated by the presence of IL-1β, the resulting level of NF-kB which can bind the DNA is larger than that available when only IFN-γ and IL-1β are present. Thus, when IFN-γ is also added, the increased level of IRF-1 binding could produce an even greater (i.e., a three-way interaction or synergy) level of stable iNOS mRNA leading to NO production. This possible qualitative description needs to be tested with a more rigorous quantitative model.

Other possible sites where the cytokines may exert effects that could impact NO production are mRNA processing and stability, iNOS translation, or posttranslational activation of the iNOS protein. One cytokine (e.g., TNF-α) could inhibit the degradation of iNOS mRNA, or increase transcription efficiency and processing accuracy of iNOS mRNA induced by other cytokines (e.g., IL-1β) (Fig. 8). We cannot exclude the possibility that one cytokine affects the conformational change from inactive iNOS protein monomer to active iNOS protein dimer. For example, one cytokine (e.g., TNF-α or IFN-γ) may enhance the production of prosthetic groups (NADPH, FAD, FMN, and BH_4) that are required for iNOS conformational change and its catalytic action for releasing NO (Fig. 8). Another possibility is that one cytokine may inhibit the degradation of iNOS protein induced by another cytokine(s) (Fig. 8). Finally, we cannot overlook potential effects of inflammatory cytokines on substrate (L-arginine) concentration (Fig. 8). An inflammatory cytokine or cytokine mixture can upregulate substrate synthesis (L-arginine) and/or substrate uptake for the iNOS enzyme (23–25). Increased L-arginine substrate may be another mechanism by which NO production could be increased and lead to synergy between cytokines. These possibilities are less likely in light of the experimental evidence relating the effects of IL-1β and IFN-γ to transcription factors. In addition, TNF-α specifically augments the effect of IL-1β, but not IFN-γ on NO production. If TNF-α exerted an effect posttranscriptionally, this would augment the effects of both IL-1β and IFN-γ.

CONCLUSION

Our study was aimed at determining the specific relationship among IL-1β, TNF-α, and IFN-γ in the induction of NO in human alveolar epithelial cells. We demonstrated that TNF-α is not capable of inducing NO production alone, but can enhance the production of NO induced by IL-1β. IFN-γ can induce NO production alone, which differs from the pattern reported for rat alveolar cells. In addition IFN-γ and IL-1β induce NO by different mechanisms and act synergistically in the production of NO. We conclude that an overall hypothesis or qualitative model of the complete NO production system in the cell must be established that includes not only transcriptional control, but also pretranscriptional and posttranscriptional control. A significant amount of work, both experimental and theoretical, is needed to fully elucidate the complex mechanism underlying cytokine-induced NO production in human lung cells.

ACKNOWLEDGMENTS

The authors thank Dr. Howard Tucker in the Department of Mathematics at the University of California, Irvine for expert statistical assistance. This work was supported by a grant from the Whitaker Foundation (WF-22310).

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