Interaction of Ethanol with Inducible Nitric Oxide Synthase Messenger RNA and Protein: Direct Effects on Autacoids and Endotoxin In Vivo 1,2

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ABSTRACT

Inducible nitric oxide synthase (iNOS) mRNA is up-regulated in vivo by dibutyryl-cAMP (db-cAMP), the purine-2y receptor agonist 2-methylthio-ATP and Escherichia coli endotoxin lipopolysaccharide (LPS). Ethanol and diethyldithiocarbamate inhibit LPS-stimulated iNOS mRNA. Their effects on db-cAMP- and 2-methylthio-ATP-stimulated iNOS mRNA remain undefined. We examined the effect of ethanol (4.5 g/kg intraperitoneal) and intratracheal diethyldithiocarbamate (5 mg/kg) on intratracheal LPS (0.6 mg/kg), db-cAMP (0.1 and 1 mg/kg) or 2-methylthio-ATP (5 mg/kg)-stimulated rat alveolar macrophage (AM) iNOS mRNA and protein, reactive nitrogen intermediates nitrite and nitrate anion (RNI) and nuclear transcription factor-κB (NF-κB) in vivo. LPS and the autacoids increased iNOS mRNA and protein in rat AM and RNI in bronchoalveolar lavage fluid and in ex vivo incubates of AM compared with these parameters in control rats (n = 6–21/group). Only LPS up-regulated TNF-α mRNA and release of TNF-α in bronchoalveolar lavage fluid and AM. Ethanol inhibited LPS stimulation of the iNOS cascade at the level of transcription but inhibited only autacoid-stimulated iNOS protein and RNI. Diethyldithiocarbamate selectively inhibited the LPS-stimulated iNOS cascade at the level of transcription. Co-administration of ethanol and diethyldithiocarbamate inhibited LPS-stimulated iNOS mRNA, protein and RNI more than either inhibitor alone but did not differ from ethanol alone on autacoid-stimulated iNOS protein or RNI. LPS increased and db-cAMP did not affect NF-κB in AM. Ethanol inhibited LPS-stimulated NF-κB. Thus, two distinct pathways exist for induction of iNOS mRNA in rat AM in vivo: an FκB pathway for LPS and cytokines inhibitable by ethanol and diethyldithiocarbamate and an NF-κB-independent pathway, refractory to inhibition by ethanol and diethyldithiocarbamate for db-cAMP and 2-mes-ATP. Finally, ethanol inhibits iNOS at the level of transcription and at the level of the enzyme.

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ABBREVIATIONS: ETOH, ethanol; i.p., intraperitoneal; i.t., intratracheal; TNF-α, tumor necrosis factor-α; LPS, Escherichia coli endotoxin lipopolysaccharide; iNOS, inducible nitric oxide synthase; NO, nitric oxide; c-ERT-PCR, competitor DNA equalized reverse transcription-polymerase chain reaction; PBS, phosphate-buffered saline; HBSS, HEPES-buffered salt solution; BH4, tetrahydrobiopterin; AM, alveolar macrophage; PMN, neutrophil; RNI, reactive nitrogen intermediates; BCA, bicinchoninic acid; PKA, protein kinase A; NF-κB, nuclear factor-κB; CREB, cAMP response element; DETC, diethyldithiocarbamate; BALf, bronchoalveolar lavage fluid; db-cAMP, dibutyryl-AMP; PMSF, phenylmethylsulfonyl fluoride; EMSA, electrophoretic mobility shift assay; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Moderate consumption of ETOH and chronic alcoholism attenuate host immunological defenses and increase animal and human susceptibility to lung infections, including tuberculosis and pneumonia (Gluckman et al., 1977; Jerrells et al., 1990). The mechanism by which ETOH suppresses pulmonary host defense is complex (Jerrells et al., 1990). ETOH has been shown to suppress up-regulation of macrophage and PMN cytokine- and oxygen-derived free radical production by bacteria, mycobacteria tuberculosis and fungi and their cell wall components (Astry et al., 1983; Greenberg et al., 1995b; Jerrells et al., 1990). ETOH also can inhibit PMN migration from the lung and circulation into the alveolar space (Astry et al., 1983; Gluckman et al., 1977). Moreover, ETOH can suppress pulmonary host defense mechanisms evoked by microorganisms or their cell wall-derived toxins by inhibiting the secretion of TNF-α from AM and its recruitment of PMN into the lung (Beutler and Grau, 1993). In addition, ETOH down-regulates signal transduction in phagocytic cells in response.
to superoxide (Dorio and Forman, 1988) and TNF-α (Nelson et al., 1989) by decreasing TNF-α receptor synthesis or the binding of TNF-α to its receptors or by up-regulating membrane transport of TNF-α (Bermudez et al., 1991; Deaciuc et al., 1992).

NO is also involved in the pulmonary host response to infection and inflammation. In addition to its ability to act as a potent endogenous bacteriostatic, bactericidal and cytotoxic agent, NO can modulate the activation and adhesion of AM and PMN within the lung and thereby affect the bactericidal activity of these phagocytic cells (Albina and Reichner, 1995; Nathan and Hibbs, 1991). Endogenous NO can also modulate both airway epithelial function and bronchiolar and pulmonary vascular smooth muscle tone in normal individuals and in patients with asthma (Yates et al., 1995). Recent studies demonstrated that cell-signaling pathways required for expression of cytokine and LPS-iNOS appear to be modulated by substances that up-regulate cAMP and stimulate P2Y receptors. Compounds that regulate the cAMP system either enhance or inhibit the ability of cytokines and LPS to up-regulate iNOS protein in cultured murine macrophages, rat mesangial and vascular smooth muscle cells and cardiac myocytes, purportedly by inhibition of the degradation of iNOS mRNA and/or protein or by stimulation of translation (Bulut et al., 1993; Hirokawa et al., 1994; Koide et al., 1993; Kunz et al., 1994). However, in vivo administration of db-cAMP and the P2Y receptor agonist 2-mes-ATP only up-regulated iNOS mRNA and enhanced LPS-mediated up-regulation of iNOS mRNA in rat AM (Greenberg et al., 1996). Thus, interference with the NO system may not only affect the response of the lung to pathogenic organisms but also inhibit the NO component of the response to medications that increase the cAMP system or stimulate P2Y receptors.

ETOH has a dual effect on production of NO by the constitutive and inducible forms of NOS. ETOH increases the synthesis of NO from the vascular endothelium of normal animals and antagonizes Escherichia coli- and LPS-mediated suppression of constitutive NOS in the adrenal gland and aortic endothelium (Greenberg et al., 1994, 1995a, 1995b; Xie et al., 1995). In contrast, acute administration of ETOH to rats inhibits LPS-mediated up-regulation of iNOS mRNA in hepatic Kupffer and endothelial cells (Spolarics et al., 1993) and in AM and PMN obtained from LPS-treated rats (Greenberg et al., 1994; Kolls et al., 1995; Xie et al., 1995). However, the effect of ETOH on inducers of iNOS other than cytokines, endotoxins and microorganisms has not been defined. This study examines the effect of acute administration of ETOH on the ability of db-cAMP and 2-mes-ATP to up-regulate iNOS mRNA and iNOS protein in rat AM in vivo.

Materials and Methods

Experimental Design

ETOH, autacoids and LPS on lung and AM iNOS and TNF-α. Male Sprague-Dawley rats (225–250 g; Hilltop Farms, Scottsdale, PA) were housed at the LSU Medical Center vivarium under a 12-h dark/light cycle and allowed food and water ad libitum. On the morning of the experiment, the rats were administered i.p. sterile PBS (4.5 mg/kg) or ETOH (4.5 g/kg). Twenty-five minutes later, the rats were anesthetized with ether, the trachea was isolated and the heart and lungs were removed, and the lung was subjected to bronchoalveolar lavage with three 10-ml washes with PBS. The BALf was analyzed for TNF-α, ETOH and NO3 and NO2 anions (RNI). The heart and lungs were removed, and the lung was subjected to bronchoalveolar lavage with three 10-ml washes with PBS. The BALf was analyzed for TNF-α and RNI. The AM were isolated from the BALf and used for determination of the total cell count, differential percentage of AM and PMN, iNOS mRNA and protein, TNF-α mRNA, NF-κB and the concentration of RNI in the ex vivo incubates of the freshly isolated AM (Greenberg et al., 1994, 1995b; Kolls et al., 1995; Xie et al., 1995). The rationale for choosing the i.t. route of administration, time period of 2 hr after LPS and i.p. administration of ETOH has been described in detail (Greenberg et al., 1994, 1995b; Kolls et al., 1995; Xie et al., 1995). The mixed β1- and β2- adrenergic agonist isoproterenol and the β2- adrenergic agonist albuterol were evaluated on the NO system of rat AM for several reasons. First, db-cAMP and 2-mes-ATP can produce changes in bronchial or pulmonary vascular smooth muscle pressure or flow, thereby affecting lung resistance and permeability. Moreover, i.t. administration of these autacoids may have stimulated extra-alveolar cells. Finally, i.t. administration of polar compounds may have a nonspecific irritant effect on the AM in the lungs. Each of these effects may potentially up-regulate the iNOS system in the rat lung. The tracheal and bronchial epithelium and airway and pulmonary vascular smooth muscle are endowed with β1- and β2- adrenoceptors, whereas the rat AM are essentially devoid of β1 adrenoceptors. Thus, the rats were given isoproterenol or albuterol by the i.t. route to account for the potential effects of the autacoids.

Effect of DETC on LPS and autacoid/NOS interaction. To examine the cell-signaling pathway involved in the inhibitory effect of ETOH on LPS- and autacoid-mediated up-regulation of the iNOS or TNF-α systems, the experiments described above were repeated in rats pretreated with DETC (5 mg/kg i.t.), an inhibitor of NF-κB (Mulsch et al., 1993). Rats were anesthetized with ether, the trachea was isolated and DETC was administered by the i.t. route. The rats were immediately given PBS (4.5 ml i.p.) or ETOH (4.5 g/kg i.p.) and allowed to awaken. Thirty minutes later, the rats were anesthetized with ether and given i.t. injections of PBS (0.5 ml), LPS (0.6 mg/kg), db-cAMP (0.1 or 1.0 mg/kg) or 2-mes-ATP (5 mg/kg). Two hr later, the rats were anesthetized, and the biochemical and molecular biological assays listed above and described immediately below were performed.

Biochemical and Molecular Biological Methods

Cell counts and differentials. Cell counts were performed on washed cells with a hemacytometer using a Motorola video system (Cole-Palmer, Chicago, IL). Differentials were performed on CytoSpin preparations stained with Diff-Quik (Baxter, McGraw Park, IL). Viability was always >95% as determined by trypan blue exclusion (Greenberg et al., 1994; Kolls et al., 1995; Xie et al., 1995).

Cell separation. The AM were isolated from the BALf of individual lavage samples using a Percoll-Ficoll gradient after initial isolation with Polymorph-Prep (Nycomed, GIBCO, Grand Island, NY). The 2-hr samples from all rats except those treated with LPS consisted of >99% pure AM. Cell viability as determined by trypan blue exclusion was >95%. Isolation and purification of these cells have been described previously (Greenberg et al., 1994; Kolls et al., 1995; Xie et al., 1995).

Assay of mRNA for iNOS and TNF-α. An aliquot of AM (2 × 106 AM/ml) was frozen in liquid nitrogen and assayed for mRNA for iNOS and TNF-α. Transcripts for iNOS and TNF-α in the AM were measured by cERT-PCR. Primer sequences for iNOS and TNF-α were iNOS-A, 5′-AATGGGACACATCCAGTCGCGGACT-3′; iNOS-B, 5′-GTGCTGGTGGTCACAGAAGTCT-3′; TNF-α-A,
5'-AGGTTGATCTTCTCTGTAGA-3'. The results were expressed as pg of iNOS or TNF-α mRNA/ng of CDNA. The method has been described in detail (Greenberg et al., 1994, 1996; Kolls et al., 1995; Xie et al., 1995).

**Measurement of iNOS enzyme by Western blot.** An aliquot of BALF (0.5 ml) containing 3 million cells was centrifuged at 1500 × g at 4°C for 10 min. The pellets were then homogenized with 0.5 ml of homogenization buffer [20 mM Tris-HCl, pH 7.5, 0.25 M sucrose, 2 mM ethylene glycol bis(α-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 2 mM EDTA, 0.02% leupeptin, 1 mM PMSF and 0.1% Triton X-100]. The homogenates were incubated for 1 hr at 4°C and centrifuged at 3000 rpm for 30 min at 4°C in a Tabletop Refrigerated TJ-9 centrifuge (Beckman Instruments, Fullerton, CA). The supernatants were stored at −20°C. The concentration of protein in the homogenates was determined by the bicinchoninic acid method (Goldschmidt and Kimmelberg, 1989). Protein samples (50 μg) were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. Proteins were electrophoretically transferred to nitrocellulose using a Semi-Dry Transfer Cell (BioRad, Hercules, CA). The transfer buffer used was 48 mM Tris-HCl and 39 mM glycine buffer (pH 9.2) containing 0.037% sodium dodecyl sulfate and 5% methanol. After blocking nonspecific sites with blocking solution containing 5% (w/v) nonfat milk and 0.05% Tween 20 in PBS (80 mM Na2HPO4, 20 mM NaH2PO4, 100 mM NaCl, pH 7.5) for 1 hr at 4°C, the nitrocellulose membrane was incubated with polyclonal anti-rat iNOS antibody (Transduction Lab, Louisville, KY) at a concentration of 1:50,000 dilution in PBS containing 5% (w/v) nonfat milk and 0.05% Tween 20 (overnight at 4°C). After washing, the membrane was incubated for 1 hr at room temperature with the secondary antibody linked to horseradish peroxidase (1:5000 dilution in 1% nonfat milk and 0.05% Tween 20 in PBS) and subsequently washed in 0.05% Tween 20 in PBS. The bound antibody on the membrane was detected by the enhanced chemiluminescence method according to the manufacturer’s instructions (Amersham, Arlington Heights, IL). Exposure times of immunoblots to Hyperfilm were 1 min. The density of specific iNOS band was quantified using Foto Touch and Sigma Gel densitometric analysis (Jandel Scientific, Sausalito, CA).

**Measurement of RNI.** Aliquots of freshly isolated AM (2 × 107/ml) were incubated in HPSS, pH 7.4, containing 128 mM NaCl, 4.9 mM KCl, 1.2 mM MgCl2, 1.6 mM CaCl2, 10 mM dextrose, 18.7 mM Na-HEPES/HEPES buffer, 1.18 mM Na2HPO4 and 130 mM l-arginine for 1 hr. The cells were immediately removed through centrifugation at 5000 × g for 15 min at 4°C, and the incubate was assayed for RNI with ozone chemiluminescence or TNF-α with the WEHI assay (see below). The RNI of plasma, BALF or ex vivo incubates of AM by the WEHI assay as described previously in detail (Greenberg et al., 1994, 1996; Kolls et al., 1995; Xie et al., 1995).

**NF-κB assay.** Nuclear extracts from AM (107/μl) were prepared according to the method of Dignam et al. (1983) as modified by Schreck and Baueuerle (1994). Briefly, the AM were washed once in cold PBS and centrifuged (10 min at 500 × g at 4°C). The pellet containing the cells was resuspended in 150 μl of buffer A (10 mM HEPES-KOH, pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 2 mM dithiothreitol, 1 mM PMSF and 4 μg/ml apronitin), incubated on ice for 15 min and homogenized by five passages through a 25-gauge needle. After centrifugation (10 min at 4°C at 6000 × g), the supernatant was centrifuged again at 4°C for 20 min at 15,800 × g. Glycerol (10% v/v) was added to the resulting supernatant, which was then analyzed by EMSA for sequestered NF-κB after treatment with deoxycholate (0.4%). The nuclear pellets were resuspended in 60 μl of buffer B [20 mM HEPES-KOH, pH 7.9, 400 mM KCl, 0.1 mM EDTA, 25% (v/v) glycerol] containing 1 mM PMSF and 4 μg/ml apronitin. The suspension was incubated on ice for 20 min, and the mixture was centrifuged (20 min at 4°C at 15,800 × g). The resulting supernatant was diluted 1/1 (v/v) with buffer D containing 0.25% (v/v) NP-40. Nuclear extracts were immediately used for EMSA or frozen in liquid nitrogen and stored at −70°C. The protein concentration of the nuclear extracts was determined according to the method of Bradford (1976) standardized with bovine serum albumin.

**EMSA.** EMSA was performed according to the manufacturer’s protocol (Promega, Madison, WI). The oligonucleotide sequence of NF-κB was 5′-AGT TGA GGG GAC TTT CCC AGG C-3′ and 3′-TCA ACT CCC CTG AAA GTC G-5′. The duplex of the κB DNA was end-labeled with [γ32P]ATP using T4 polynucleotide kinase. Nuclear extracts (2.5–10 μg) were preincubated in 20-μl reaction mixtures containing 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 0.2 mM dithiothreitol, 5% to 10% glycerol and 2 μg of poly(dI-dC). After 5 min at room temperature, 2 μl of [γ32P]ATP labeled oligonucleotide duplex probe was added, and the incubation was continued for another 20 min. In competition experiments, the unlabeled competitor DNA (50–100-fold molar excess) was added 5 min before the addition of the radiolabeled probe. For supershift analysis, 1 μg of each antibody was added to the reaction mixtures immediately after the addition of the radiolabeled probe. After the binding reaction, 2 μl of 10% gel loading buffer was added, and the reaction was subjected to a nondenaturing 5% acrylamide gel (in 0.5×TBE running buffer at 200 V for ~2 hr). The gels were dried, exposed to a storage phosphor screen and scanned on a PhosphorImager. Data were expressed as a percent of the nuclear extracts obtained from PBS-treated rats.

**Statistical Analysis**

Each experiment was replicated with 6 to 21 rats per group. Data were analyzed with analysis of variance for a randomized complete block or completely random sample design. Biochemical data were analyzed with multivariate analysis of variance and multivariate analysis of covariance. Differences between and among mean values were analyzed with Tukey’s procedure and Dunnett’s test. A value of P ≤ .05 was accepted for statistical significance of mean differences.

**Results**

**Plasma concentrations of ethanol.** The plasma concentration of ETOH was measured in each of the four groups of rats pretreated with PBS, ETOH, DETC or DETC in combination with ETOH. The plasma concentrations of ETOH were 2.78 ± 0.37 and 3.04 ± 0.56 (mM ± S.E.M., n = 6–27), respectively, when obtained from the PBS- and DETC-pretreated rats and did not differ (P > .34). The plasma concentrations of ETOH obtained from the ETOH- and ETOH-and-DETC-pretreated rats were 55.6 ± 2.93 and 54.8 ± 3.72 (mM ± S.E.M., n = 6–27), respectively, and also did not differ from each other (P > .39).

**BALF cell counts and differentials.** The content of BALF obtained from control rats given PBS or ETOH consisted of 99% to 100% AM, was essentially devoid of PMN (table 1) and did not differ from that obtained from untreated rats (data not shown). Treatment of rats with LPS increased the total number of cells in the BALF (the sum of the AM and PMN) by increasing the number of recoverable AM and by recruitment of PMN into the lung. In contrast, db-cAMP, 2-mes-ATP, isoproterenol and albuterol did not stimulate recruitment of PMN into the alveolar space of the lung (table 1). ETOH suppressed LPS-mediated stimulation of PMN recruitment.
into the alveolar space and subsequently the BALf content of AM and PMN (table 1). As shown previously, DETC alone (Greenberg et al., 1996) did not affect the BALf content of AM and PMN obtained from PBS-treated rats but attenuated LPS-induced increases of AM and recruitment of PMN into the alveolar space, thereby decreasing the total cell number without affecting the relative distribution of AM and PMN. Treatment of rats with the combination of DETC and ETOH did not affect the BALf content of AM and PMN obtained from PBS-treated rats but produced greater suppression of LPS-induced increases of the AM and PMN recovered from BALf than either pretreatment alone (table 1).

Effects of ETOH on LPS and autacoid stimulation of iNOS and TNF-α systems. LPS, db-cAMP (0.1 and 1 mg/kg) or 2-mes-ATP up-regulated the AM content of iNOS mRNA within 2 hr after their i.t. administration to the rats (fig. 1, top). The content of iNOS mRNA generated in AM obtained from rats treated with db-cAMP (0.1 and 1.0 mg/kg) was dose related. The iNOS mRNA content of AM produced by db-cAMP (1 mg/kg) and 2-mes-ATP did not differ from that produced by LPS. Pretreatment of rats with ETOH attenuated LPS-mediated up-regulation of iNOS mRNA but did not affect that produced by db-cAMP or 2-mes-ATP (fig. 1, top). The mRNA for iNOS was undetectable in the AM obtained from rats treated with PBS, isoproterenol or albuterol (fig. 1, top). Basal levels of TNF-α were present in the AM obtained from PBS-treated rats. Only LPS up-regulated the mRNA for TNF-α obtained from the rat AM. Pretreatment of rats with ETOH did not affect LPS-mediated up-regulation of TNF-α mRNA in the rat AM (fig. 1, middle). Within 2 hr after their i.t. administration to the rats, LPS, db-cAMP and 2-mes-ATP up-regulated iNOS protein of the AM. The content of iNOS protein generated in AM obtained from rats treated with db-cAMP (0.1 and 1.0 mg/kg) was dose dependent. However, the iNOS content of the AM obtained from rats treated with db-cAMP or 2-mes-ATP was less (P < .05) than that produced by LPS (fig. 1, bottom), despite little difference in their iNOS mRNA content (fig. 1, top). Pretreatment of rats with ETOH attenuated LPS-induced increases of the iNOS protein content of the rat AM, as would be expected after inhibition of iNOS mRNA. Despite its inability to inhibit db-cAMP- and 2-mes-ATP-stimulated iNOS mRNA, ETOH inhibited db-cAMP- and 2-mes-ATP-induced increases of iNOS protein in rat AM (fig. 1, bottom). iNOS protein was undetectable in the AM obtained from the rats treated with PBS, ETOH, isoproterenol or albuterol (fig. 1, bottom).

![Image](https://via.placeholder.com/150)

**Fig. 1.** Effect of pretreatment with PBS (4.5 ml/kg i.p.) or ETOH (4.5 g/kg i.p.) on rat AM iNOS mRNA (A), TNF-α mRNA (B) or iNOS protein (C) produced by i.t. administration of PBS (0.5 ml), LPS (0.6 mg/kg), db-cAMP (0.1 and 1 mg/kg), 2-mes-ATP (5 mg/kg), albuterol (0.5 mg/kg) or isoproterenol (0.2 μg/kg). PBS and ETOH were given 30 min before i.t. administration of LPS or the autacoids. AM were obtained 2 hr after i.t. administration of drugs. The ordinates are the mean and S.D. of pg of mRNA/ng of cDNA corrected for background (A and B) or gel density units of iNOS protein (C). Vertical lines are the S.D. from 5 to 17 rats/mean. *, Responses in the ETOH- and PBS-pretreated rats differ (P < .05). †, Responses for each agonist in the DETC-, ETOH- or ETOH-plus-DETC-treated rats differ from that of the agonist in PBS-pretreated control rats (P < .05).

<table>
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<th>Pretreatment drug</th>
<th>PBS (4.5 ml/kg)</th>
<th>ETOH (4.5 g/kg)</th>
<th>DETC (5 mg/kg)</th>
<th>DETC + ETOH</th>
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<td>(cell count)</td>
<td>(cell count)</td>
<td>(cell count)</td>
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<td>13 ± 1.4</td>
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<td>Albuterol</td>
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<td>20 ± 0.5</td>
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* P < .05 from PBS control.

b P < .05 from LPS in PBS-pretreated rats.
The concentrations of TNF-α in rat plasma, BALf and the ex vivo incubates of AM were below the limits of detection of the WEHI assay in rats treated with PBS (4.5 ml/kg i.p.) or ETOH (4.5 g/kg i.p.) 30 min before i.t. administration of PBS, LPS, db-cAMP, 2-mes-ATP, albuterol or isoproterenol (data not shown) (Greenberg et al., 1995a, 1995b; Xie et al., 1995). The concentrations of TNF-α increased in the BALf and ex vivo incubates of AM only in rats treated with LPS. Pretreatment of rats with ETOH attenuated LPS-mediated increases in the concentrations of TNF-α (ng/ml, mean ± S.D.) in BALf 43.8 ± 9.3 (n = 19) to 20 ± 7.1 (n = 20) (P < .05) and in the ex vivo incubates of AM 2.92 ± 0.9 (n = 19) to 1.8 ± 0.4 (n = 20) (P < .05).

A basal level of RNI existed in the BALf of rats treated with PBS and ETOH (fig. 3, top). The RNI of the ex vivo incubates of AM obtained from PBS- and ETOH-treated rats did not differ from that of the buffer in the absence of cells (Greenberg et al., 1995a, 1995b). Pretreatment of rats with LPS, db-cAMP or 2-mes-ATP increased RNI levels in BALf and the ex vivo incubates of AM (fig. 3, top and bottom). However, the increase in RNI induced by db-cAMP and 2-mes-ATP was smaller than that found in the BALf and ex vivo incubates of AM obtained from the LPS-treated rats (fig. 3, top and bottom). Isoproterenol and albuterol did not increase RNI in the BALf or ex vivo incubates obtained from rat AM. Pretreatment of rats with ETOH attenuated the RNI levels in the BALf and the ex vivo incubates obtained from LPS, db-cAMP and 2-mes-ATP treated rats (fig. 3, top and bottom).

**Effect of DETC on iNOS mRNA, protein and RNI.** Similar to the effects seen with ETOH, pretreatment of rats with DETC selectively inhibited LPS-mediated up-regulation of iNOS mRNA and protein compared with those parameters in rats pretreated with LPS, db-cAMP or 2-mes-ATP (fig. 4). The magnitude of DETC-mediated inhibition of LPS-induced up-regulation of iNOS mRNA did not differ from that obtained after pretreatment with ETOH, whereas DETC-mediated inhibition of iNOS protein was somewhat greater (P < .05) than that obtained with ETOH. However, when rats were pretreated with the combination of DETC and ETOH, the percent inhibition of LPS-stimulated iNOS mRNA and iNOS protein was greater (P < .05) than that obtained with either pretreatment alone (fig. 4, top and bottom). In contrast, db-cAMP and 2-mes-ATP-stimulated increases of rat AM iNOS protein were equally inhibited 2 hr after concurrent administration of DETC and ETOH compared with the magnitude of inhibition produced by ETOH alone (fig. 4, bottom). A representative series of cERT-PCR gels showing the effect of DETC or DETC in combina-
Pretreatment of rats with DETC selectively inhibited LPS-mediated up-regulation of RNI levels in the BALF and ex vivo incubates of AM compared with those parameters in rats pretreated with DETC and subsequently given db-cAMP or 2-mes-ATP (fig. 6). The magnitude of DETC-mediated inhibition of LPS-induced increases of RNI in the BALF and ex vivo incubates of AM was significantly less than that obtained after pretreatment of rats with ETOH alone (P < .05). Responses for each agonist differ from that in the DETC-treated group (P < .05). Responses for each agonist in the presence of ETOH and DETC differ from that for the agonist in the presence of ETOH alone (P < .05).

Effect of DETC on LPS-mediated up-regulation of TNF-α. Pretreatment of rats with DETC did not affect LPS-mediated up-regulation of TNF-α mRNA in rat AM or TNF-α protein in BALF and ex vivo incubates of AM and the ability of ETOH to attenuate these effects of LPS (fig. 7).

Effect of LPS and db-cAMP on NF-κB. Basal NF-κB DNA binding activity has been described in isolated monocytes and macrophages (Choi et al., 1995; Trebilcock and Ponnappan, 1996). We also found slight activation of NF-κB DNA binding activity in the nuclear extract and cytosolic homogenates isolated from PBS-treated rats. The amount of NF-κB in the nuclear extracts of AM obtained from the rats varied among the individual rats within the control group but was consistently and significantly lower than that seen after induction with LPS. Therefore, the NF-κB in nuclear extracts obtained from rats administered LPS, db-cAMP and ETOH, alone or in combination, were expressed as a percent of the levels of NF-κB obtained in the PBS-treated rats. We were unable to test 2-mes-ATP in this assay because it has been commercially unavailable since November 1, 1996. The NF-κB binding activity of the nuclear extracts obtained from the AM of LPS-treated rats increased in a time-dependent manner compared with that obtained from PBS-treated rats (data not shown). Thirty minutes after administration of LPS, the binding activity of NF-κB was significantly increased and reached its peak value at 2 hr after drug admin-
The autacoid-induced up-regulation of iNOS mRNA was refractory to inhibition by acute in vivo administration of ETOH and DETC, which attenuated LPS- and cytokine-mediated up-regulation of iNOS mRNA (Greenberg et al., 1994; Kolls et al., 1995; Syapin, 1995; Xie et al., 1995). This appeared to be explained by the finding that the autacoid-induced up-regulation of iNOS mRNA was accompanied by low-level stimulation of NF-κB binding activity, whereas LPS significantly up-regulated this transcription factor, which is essential for LPS-mediated up-regulation of iNOS mRNA (Ayoubi and Van de Ven, 1996; Mandrekar et al., 1996; Mulsch et al., 1993; Wang et al., 1996). Also, ETOH attenuated LPS-mediated up-regulation of NF-κB but did not affect the small increases produced by db-cAMP and 2-mes-ATP. This could explain why the autacoid-mediated induction of iNOS mRNA was refractory to inhibition by ETOH and DETC. In addition, significantly less iNOS protein and RNI were formed from equivalent amounts of iNOS mRNA in the AM obtained from the autacoid-treated rats compared with that obtained from the AM of the rats treated with LPS. Finally, the autacoid-induced up-regulation of iNOS mRNA was not part of an elicited inflammatory response because PMN recruitment into the lung and up-regulation of TNF-α mRNA within the AM and release of TNF-α from the AM were absent (Beutler and Grau, 1993; Jerrells et al., 1990). These data are consistent with the conclusion that the two distinct cell-signaling pathways exist in rat AM for induction of iNOS mRNA by LPS and autacoids (Denlinger et al., 1996; Kleinert et al., 1996). The cell-signaling pathway used by db-cAMP and 2-mes-ATP for induction of iNOS mRNA is independent of activation of NF-κB and of the inflammatory response of the lung, whereas that for LPS-stimulated iNOS mRNA is dependent on up-regulation of NF-κB and is associated with activation of the nonspecific host defense mechanisms of the lung. The last new finding of this study was that ETOH also acted at a posttranscriptional site or sites to decrease the amount of iNOS protein and RNI in AM. This action of ETOH on iNOS protein in AM was unmasked as a result of its inability to inhibit autacoid-induced iNOS mRNA.

**ETOH selectively inhibits LPS-stimulated iNOS mRNA and NF-κB.** Using isolated cells in culture, it has been postulated that at least two distinct cell-signaling pathways exist for induction of iNOS mRNA in vitro based on studies conducted on isolated tissues and cells in culture. One pathway is activated by some cytokines and LPS, whereas the second pathway is activated by interleukin-1β and other agents unrelated to inflammatory cytokines and lipids (Denlinger et al., 1996; Kleinert et al., 1996; Oddis et al., 1995; Tonetti et al., 1994). A third potential pathway may also exist, which is activated by tissue plasminogen activator (Kleinert et al., 1996). Our data support the existence of at least two distinct cell-signaling pathways for induction of iNOS mRNA in AM in vivo. Acute administration of ETOH attenuates lung host defense mechanisms in part by inhibition of LPS and cytokine-stimulated induction of NO at the level of transcription of iNOS mRNA (Greenberg et al., 1994, 1995b; Xie et al., 1995). LPS-induced up-regulation of iNOS mRNA is dependent on its ability to increase the transcription factor NF-κB (Kleinert et al., 1996; Mandrekar et al., 1996; Wang et al., 1996; Xie et al., 1994). This study also demonstrated that in vivo administration of LPS increased NF-κB nuclear binding activity within the AM. ETOH sup-
pressed LPS-mediated induction of NF-κB and iNOS mRNA without affecting basal NF-κB binding activity. Because DETC, a documented inhibitor of LPS- and cytokine-mediated stimulation of the transcription factor NF-κB (Mulsch et al., 1993), mimicked the effect of ETOH on LPS-mediated up-regulation of iNOS mRNA in cultured cells, we can conclude that ETOH inhibited LPS-mediated up-regulation of iNOS mRNA at the level of transcription by preventing LPS-induced stimulation of the transcription factor NF-κB. However, the refractoriness of both db-cAMP- and 2-mes-ATP-induced iNOS mRNA to inhibition by ETOH and DETC is consistent with the conclusion that ETOH and DETC sensitive to inhibition by ETOH and DETC. The second pathway appears to be activated by db-cAMP and 2-mes-ATP. It is not associated with significant activation of NF-κB and is refractory to inhibition by DETC and ETOH, suggesting it is independent of the transcription factor NF-κB.

**Speculation on the mechanism of db-cAMP and 2-mes-ATP induction of iNOS mRNA.** This study cannot define the molecular mechanism of db-cAMP- or 2-mes-ATP-mediated up-regulation of iNOS mRNA. However, some limited speculation is warranted based on published data on the regulation of transcription of iNOS mRNA. The mouse iNOS gene contains binding sites for various transcription factors (Xie et al., 1993), but there are no binding sites for the CREB. Even without the CREB sites on rat iNOS gene db-cAMP, cAMP and 2-mes-ATP may stimulate other transcription factors or promoters, which in turn can activate the iNOS gene (Ayoubi and Van De Ven, 1996). Alternatively, a rapid increase and decrease in iNOS mRNA may occur through the action of a cycloheximide-inhibitable protein (Evans et al., 1994), which prolongs the lifetime of iNOS mRNA (Hattori and Gross, 1995). This protein may be a promoter or enhancer of iNOS transcription. Thus, db-cAMP and 2-mes-ATP may stimulate protein kinase A-mediated phosphorylation of this protein, which activates a stimulator of, or
inhibits a repressor of iNOS mRNA transcription (Brune and Lapetina, 1991). Further studies are required to elucidate the mechanism by which db-cAMP and 2-mes-ATP up-regulate transcription of iNOS mRNA.

**Differences between in vivo and in vitro studies.** In vivo administration of 2-mes-ATP to rats up-regulated iNOS mRNA, iNOS protein in the AM and generation of RNI in BALf and ex vivo incubates of AM compared with rats given PBS. This is in contrast to the cell culture studies, which demonstrate that ATP and 2-mes-ATP do not affect or inhibit iNOS mRNA in RAW-264.7 cells, murine peritoneal macrophages and glioma cells and astrocytes while attenuating LPS- and cytokine-mediated up-regulation of iNOS mRNA and RNI production (Denlinger et al., 1996; Murphy et al., 1995). Moreover, although ETOH inhibits up-regulation of iNOS mRNA and iNOS protein by interleukins in vivo ETOH enhances the production of NO by iNOS in response to interleukin-1β in cultured vascular smooth muscle cells (Durante et al., 1995). The factors that can explain these differences include the dose or concentration of ETOH used, the inducer of iNOS used (LPS, db-cAMP or 2-mes-ATP or interleukin-1β) and the cell types studied. However, the most important difference may be the difference between in vivo and ex vivo models compared with the study of cells in culture. The use of cultured cell lines and freshly isolated cells incubated in culture medium may modify the cell signaling pathways used to up-regulate transcription of iNOS mRNA and its ability to translate the message into iNOS protein in vivo or in vitro (Sirsjo et al., 1994). Many cell lines and cells in culture exhibit phenotypic and genotypic transformations that result in qualitatively different responses to cytokines, autacoids and drugs in vivo and in cell culture (Durante et al., 1994; Greenberg, et al., 1994; Schroder et al., 1987). Also, the cell signaling pathway for induction of iNOS differs between isolated aortic strips and aortic smooth muscle cells in culture (Sirsjo et al., 1994). Thus, the in vivo pathway used to up-regulate iNOS in AM by db-cAMP, 2-mes-ATP and possibly even LPS and their interaction with ETOH may differ in vivo from those pathways used in vitro in cultured cells.

**Equivalent iNOS mRNA results in different amounts of iNOS protein.** For equal amounts of iNOS mRNA produced by LPS, db-cAMP and 2-mes-ATP, significantly smaller amounts of iNOS protein was produced in the AM obtained from db-cAMP-pretreated rats than that produced by LPS or 2-mes-ATP. The cause of this difference in translation of iNOS protein remains to be examined. However, published studies and preliminary data provide some potential explanations for these differences. First, two distinct isoforms of iNOS have been found to be produced in the rat kidney by iNOS mRNA (Mohaupt et al., 1994). Thus, it is possible that db-cAMP may induce an isozyme of iNOS distinct from that isozyme of iNOS protein induced by LPS or 2-mes-ATP. This suggests the putative isozymes may be differentiated by or respond differently to the iNOS polyclonal antibody. Alternatively, dimerization of the monomeric subunits of iNOS protein are required for the post-translational activation of iNOS activity (Cho et al., 1995). Among the factors promoting dimerization are arginine, heme and BH4. LPS increases arginine transport and the up-regulation of BH4 in macrophages (Griffith and Stuehr, 1995; Gross and Levi, 1992). di-cAMP does not increase the uptake of arginine or the synthesis of BH4 in macrophages (Forstermann et al., 1995; Griffith and Stuehr, 1995; Hua et al., 1996). Thus, we can also speculate that db-cAMP may delay the dimerization of iNOS because of its inability to up-regulate the transport and synthesis of arginine and BH4, respectively, in the AM. Alternatively, although db-cAMP-induced up-regulation of iNOS mRNA reaches peak levels 2 hr after i.t. administration of this autacoid, the initial rate of mRNA formation is dose dependent and slower than that seen with LPS. This may also explain the significant difference in iNOS protein between the low and high doses of db-cAMP. Further studies are required to test these postulates.

**ETOH inhibits iNOS protein.** Previous studies demonstrated that acute administration of ETOH in vivo and in vitro inhibited LPS-mediated up-regulation of iNOS mRNA (Greenberg et al., 1994; Kolls et al., 1995; Syapin, 1995; Xie et al., 1995). Because iNOS enzyme is regulated at the level of transcription (Forstermann et al., 1995; Griffith and Stuehr, 1995), it was impossible to determine whether ETOH inhibited post-transcriptional processes or translation of iNOS. The finding that ETOH did not affect db-cAMP- or 2-mes-ATP-mediated up-regulation of iNOS mRNA provided a suitable model to evaluate the effects of ETOH on the synthesis of iNOS protein. Pretreatment of rats with ETOH attenuated db-cAMP- and 2-mes-ATP-mediated up-regulation of iNOS protein obtained from rat AM and RNI levels in BALf and ex vivo incubates of AM. Because db-cAMP- and 2-mes-ATP-stimulated iNOS mRNA were not affected by ETOH, it is unlikely that ETOH acted at a post-transcription site regulating the degradation or stability of the iNOS mRNA. However, the decreased content of iNOS protein obtained from the rat AM may have resulted from an inhibitory effect of ETOH on the process of translation, the stability of iNOS protein itself or the ability of the iNOS monomers to dimerize and form the active iNOS protein (Cho et al., 1995). Thus, ETOH not only inhibits the transcription process for LPS- and cytokine-induced iNOS mRNA but also inhibits the up-regulation of iNOS protein for each of the agonists tested independent of its ability to suppress gene expression for iNOS. However, our study cannot rule out the possibility that when given in vivo, ETOH may have an additional inhibitory effect on the enzymatic activity of iNOS.

**Lung inflammation is not required for up-regulation of iNOS mRNA.** Nitric oxide is an important component of the pulmonary host response to infection and inflammation because of its contribution to the overall bactericidal and viral killing activity of AM and PMN (Nathan and Hibbs, 1991). Maximal induction of iNOS requires the simultaneous action of several cytokines (Albina and Reichner, 1995; Nathan and Hibbs, 1991). The AM become phagocytic when activated by bacterial endotoxins, which then stimulate AM production of cytokines such as TNF-α, interleukin-1β, interleukin-6 and interferon-γ. The concentration of each cytokine reaches its peak value at different times after the inflammatory challenge, with peak levels of the cytokines ranging from 90 min to 2 hr for TNF-α to 12 to 24 hr for interferon-γ. In most experimental models, TNF-α is released before other cytokines, promulgating the hypothesis that secretion of TNF-α is the priming factor for the cytokine cascade, which is essential for the host defense response (Beutler and Grau, 1991).
Although this paradigm was extended to LPS-mediated up-regulation of NO derived from iNOS (Albina and Reichner, 1995; Nathan and Hibbs, 1991), subsequent studies demonstrated that LPS-mediated up-regulation of iNOS mRNA and RNI production and stimulation of TNF-α occurred by parallel and independent mechanisms and that TNF-α was not required for LPS-induced up-regulation of iNOS mRNA or its suppression by ETOH in the rat lung (Greenberg et al., 1995a; Kolls et al., 1995; Xie et al., 1995). The lack of dependence of iNOS mRNA transcription on TNF-α is clearly shown in this study with the agonists db-cAMP and 2-mes-ATP because these compounds up-regulated the former without affecting TNF-α. This and the absence of PMN infiltration into the lung may reflect the absence of an effect of db-cAMP and endogenous cAMP on NF-kB (Hecker et al., 1996). Thus, in vivo activation of iNOS mRNA in rat AM by db-cAMP and 2-mes-ATP does not elicit activation of the inflammatory response characteristic of that produced by LPS, microbiological agents and related cytokines. This suggests the possibility that cAMP and purine nucleotides may act as a selective endogenous modulators of the iNOS system in the absence of inflammation, in vivo. This may have significant implications in both research and therapeutics. First, the finding of iNOS mRNA or iNOS protein in lung and myocardial tissue of patients with asthma or heart failure in the absence of overt signs of inflammation may not reflect the role of NO in the pathogenesis of these disease processes but rather the effect of treatment with drugs that elevate cAMP or stimulate purinergic receptors in these tissues (Yates et al., 1995). In addition, these findings have potential clinical application for the treatment of chronic lung diseases, such as pulmonary hypertension or asthma, and upper and lower respiratory tract infections in which drugs that elevate cAMP or stimulate P2y receptors may be given by inhalation or aerosol to elevate endogenous NO without the deleterious effects of simultaneous activation of the cyclooxygenase or the inflammatory response.

Conclusions. We conclude that db-cAMP-, 2-mes-ATP- and LPS-mediated up-regulation of iNOS mRNA obtained from rat AM in vivo represents at least two distinct cell signaling pathways. The first is a cytokine- and LPS-stimulated pathway, involves stimulation of the transcription factor NF-kB, is inhibited by ETOH and DETC and is associated with activation of the nonselective host defense inflammatory response within the lung. The second pathway can be activated by db-cAMP and 2-mes-ATP, does not involve activation of NF-kB and so is refractory to inhibition by ETOH or DETC and is not associated with activation of the inflammatory response in the lung. We speculate that db-cAMP produced less iNOS protein than LPS for equivalent amounts of iNOS mRNA because of the difference in the rate of formation of iNOS mRNA, which may result from the different cell-signaling pathways used by these agonists We also conclude that ETOH inhibits LPS-, db-cAMP- and 2-mes-ATP-mediated up-regulation of iNOS enzyme by either inhibiting the translation or promoting the degradation of iNOS protein. Finally, if the cAMP-iNOS and 2-mes-ATP-iNOS interactions are found in humans, ETOH may modulate the ability of drugs, endogenous hormones or autacoids to generate iNOS. These may account for some of the beneficial and deleterious effects of ETOH in humans.

References


Choi, A. M. K., Sylvester, S., O’Toole, L. and Noelke, N. J.: Molecular mechanisms of cytokine-mediated up-regulation of iNOS enzyme by either inhibiting the translation or promoting the degradation of iNOS protein. Finally, if the cAMP-iNOS and 2-mes-ATP-iNOS interactions are found in humans, ETOH may modulate the ability of drugs, endogenous hormones or autacoids to generate iNOS. These may account for some of the beneficial and deleterious effects of ETOH in humans.


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