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An *in Vivo* Cytokine and Endotoxin-Independent Pathway for Induction of Nitric Oxide Synthase II mRNA, Enzyme, and Nitrate/Nitrite in Alveolar Macrophages

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Dibutyryl cyclic AMP (DB-cAMP, 0.1 and 1.0 mg/kg) and the purine-2-receptor agonist methyl-thio-ATP (MT-ATP mg/kg) given by intratracheal (i.t.) administration to rats two hr before bronchoalveolar lavage (BAL) increased iNOS mRNA to be equal to or greater than that produced by i.t. LPS, without eliciting neutrophil infiltration into the alveolar space or the upregulation of tumor necrosis factor alpha (TNFα). Translation of DB-cAMP and MT-ATP-stimulated iNOS mRNA into protein and activation of iNOS to produce RNI was slower than that resulting from LPS-stimulated iNOS mRNA. Diethyldithiocarbamate (5 mg/kg, i.t.) a sequestrant of reactive oxygen intermediates and an inhibitor of NFkappaB attenuated LPS-induced upregulation of iNOS mRNA without affecting that produced by DB-cAMP or MT-ATP. We conclude that an LPS and cytokine-independent pathway of transcription of iNOS mRNA exists *in vivo*, which can be directly activated by DB-cAMP and purine-2 receptor stimulation. It is possible that the increase in iNOS found in asthmatic patients and those with other diseases that are treated with drugs which affect the cAMP and purine systems may be iatrogenic rather than pathogenetic in origin.

Inducible nitric oxide synthase (iNOS; EC 1.14.13.39) is calcium-independent and produces a high output of nitric oxide (NO). Inducible NOS, an isoform of the NOS family of enzymes, is usually absent in resting cells and can be induced by cytokines and bacterial cell wall products such as the interleukins, tumor necrosis factor alpha (TNFα) and endotoxin lipopolysaccharide (LPS) (1). As such, induction of iNOS is primarily regulated at the level of gene transcription (2). Current evidence suggests that adenosine triphosphate and cyclic AMP act synergistically with LPS to upregulate iNOS protein in the RAW-264.7 murine macrophage cell line (3), rat mesangial cells (4) and cardiac myocytes (5) in culture by enhancement of translation or by inhibition of the degradation of NOS protein. Moreover, prolonged incubation of murine fibroblasts with cyclic AMP in culture can induce iNOS protein and mRNA (6) while suppressing LPS-induced upregulation of iNOS mRNA and the production of NO in isolated astrocytes (7) and murine macrophages (3,8) in cell culture. These data suggest that an alternate pathway for induction of iNOS independent of LPS or cytokines (6). Peptide growth factors in culture medium, and the cell culture process itself, can cause many enzymes and proteins to revert to their fetal phenotype (9,10). Moreover, many cell lines and cells in culture exhibit phenotypic and genotypic transformations which may limit the extrapolation of data obtained to the intact cells from which they were derived, *in vivo* (9,10). Thus, the *in vitro* effects of these autacoids on iNOS gene expression or enzymatic activity may not be expressed *in vivo*.

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The potential existence of alternate pathways for regulation of iNOS at the level of transcription *in vivo* takes on clinical significance in diseases such as asthma and restrictive lung diseases, where localized induction of iNOS may provide long acting bronchodilation without significant effects on the systemic circulation. Moreover, it is well established that a significant number of patients exhibit the systemic inflammatory response syndrome or respiratory distress syndrome, in which nitrate and nitrite levels are elevated, without any evidence of bacterial infection, endotoxemia or significant elevation of cytokines (11). This study was designed to determine whether cyclic AMP and 5-methylthio-ATP (MT-ATP), a purine-2 receptor agonist, *in vivo,* can directly upregulate the iNOS system in rat alveolar macrophages (AM). The data presented are the first to show that *in vivo* administration of these autacoids upregulate iNOS mRNA, iNOS protein and nitrite and nitrate (RNI) independent of LPS and TNFα, *in vivo.*

METHODS AND MATERIALS

Animals. Intratracheal (i.t.) administration of drugs into the lung and evaluation of the AM at 2 hr post injection were chosen because TNFα, the initial cytokine elicited by i.t. administration of LPS reaches its peak at this time period but remains compartmentalized within the lung and does not activate the systemic cytokine cascade (12). Moreover, the i.t. route of administration limits the interaction of the autacoids to the resident cells within the alveolar space, thereby eliminating the complexity of data interpretation resulting from the activation of circulating monocytes and macrophages and other cytokines. Conventional male Sprague Dawley rats (225-250g), obtained from Hilltop Farms (Scottsdale, PA), were housed at the vivarium of the LSU Medical Center at New Orleans under a 12 hr. dark/light cycle and were allowed food and water *ad libitum.* On the morning of the experiment the rats were anesthetized with ether, the trachea was isolated and the animals were administered either sterile saline (PBS, 0.5 ml) or Escherichia coli endotoxin (0.6 mg/kg, E. coli LPS 026:B6 from Difco, Detroit, MI), or DB-cAMP (0.1 or 1 mg/kg, RBI Inc, Cleveland, OH) or methyl-thio-ATP (5 mg/kg, RBI Inc, Cleveland, OH) alone or 30 min after i.t. administration of diethyldithiocarbamate (5 mg/kg) (Alexis Biochemical, San Diego CA). The neck wounds were closed and the animals allowed to recover. At two hr. after i.t. administration of PBS, LPS, or the autacoids the rats were anesthetized with ether, a thoracotomy was performed and blood was obtained by cardiac puncture for analyses of TNFα and NO2- and NO3- (reactive nitrogen intermediates, RNI). The heart and lungs were removed and the lung subjected to bronchoalveolar lavage (BAL) with 30 ml of sterile PBS. The BAL fluid was analyzed for TNFα and RNI. The AM and recruited neutrophils (PMN) were isolated from the BAL fluid for cell counts, mRNA and protein for TNFα and iNOS, and *ex vivo* RNI production by the freshly isolated AM (13-15).

Cell count and differential. Cell counts were performed on washed cells on a hemacytometer using a Motorola video system (Cole-Palmer, Chicago, Illinois. Differentials were performed on cytospin preparations stained with Diff-Quik (Baxter, McGraw Park, IL).Viability was always $>$ 95% as determined by trypan blue exclusion.

Cell separation. The AM were isolated from the BAL fluid of individual lavage samples using Polymorph-Prep (Nycomed, Gibco, Grand Island, NY). The two hr samples were greater than 99% pure AM. Cell viability as determined by trypan blue exclusion was greater than 95%. Isolation and purification of these cells have been described previously in detail (13-15). Freshly isolated AM (10^6 cells/0.5 ml) from each of the experimental groups were either immediately frozen in liquid nitrogen and assayed for mRNA for iNOS, immediately homogenized and assayed for iNOS protein or incubated in HEPES buffered salt solution (HPSS, pH 7.4) containing (mM): NaCl (128), KCl (4.9), $MgCl_2$ (1.2), $CaCl_2$ (1.6), dextrose (10), NaHEPES/HEPES buffer (18.7), NaH_2PO_4 (1.18) and l-arginine (130) for 1 hr. The cells were immediately removed by centrifugation at 5000 $\times$ g for 15 min at 4°C and the incubate assayed for nitrite and nitrate with ozone chemiluminescence or TNFa with the WEHI (13-15). In some experiments the HPSS contained N6-iminoethyl-L-ornithine (LNIO) (100 μM) (Alexis Biochemical, San Diego, CA) to inhibit RNI production {15}.

Measurement of RNI. Plasma, BAL fluid or AM incubates (10-50 μl) were added to 200 ml of a reducing solution (2.3% vanadium chloride in 2 N HCl at 98°C) under a stream of ultrapure nitrogen gas. The nitrate was converted to nitrite which was subsequently converted to free NO. Determination of the NO formed from RNI was made by measurement of the specific chemiluminescence resulting from the reaction of NO with ozone using a Dasibi Model 821 Nitric Oxide-NOX Analyzer (Dasibi Environmental Inc., Glendale CA). Conversion of standard solutions of nitrite to NO was $>$ 99% whereas conversion of nitrate to NO was 94-96% when compared to calibrated standards of NO gas (13-15).

Assay of mRNA for iNOS and TNFα. Transcripts for iNOS and TNFα were measured by cDNA equalized reverse transcription polymerase chain reaction (cERT-PCR) in lavaged cells as previously described (13-16). Primer sequences for iNOS and TNFα were as follows: iNOS-A: 5′-AATGGCAACATCAGGTCGGCCATCACT-3′, and iNOS-B: 5′-GCTGTGTGTCACAGAAGTCTCGAACTC-3′.; TNFα-A: 5′-AAGTTCCCAAATGGCC TCC- CTCTC ATC-3′ and

TABLE I
Effect of i.t. Administration of PBS, DB-cAMP, MT-ATP, and LPS on Indices of Lung Inflammation

Parameter	PBS (0.5 ml/kg)	DB-cAMP (0.1 mg/kg)	DB-cAMP (1 mg/kg)	MT-ATP (5 mg/kg)	LPS (0.6 mg/kg)
Cell $\times 10^6$	16.2 ± 1.3	17.1 ± 0.7	16.5 ± 0.9	16.6 ± 1.1	23.2 ± 2.1*
AM $\times 10^6$	16.1 ± 1.3	17.0 ± 0.6	16.4 ± 0.9	16.5 ± 1.1	14.9 ± 1.0
PMN $\times 10^6$	0.13 ± 0.09	0.09 ± 0.09	0.05 ± 0.04	0.13 ± 0.06	8.2 ± 1.9*
AM %	99.2 ± 0.69	99.4 ± 0.5	99.6 ± 0.03	99.2 ± 0.37	64.7 ± 4.6*
PMN %	0.08 ± 0.69	0.59 ± 0.5	0.36 ± 0.3	0.80 ± 0.37	35.3 ± 4.6*
TNFα mRNA	0.05 ± 0.02	0.06 ± 0.03	0.05 ± 0.04	0.05 ± 0.03	0.42 ± 0.13*
BALf TNFα ng	0.063 ± 0.021	0.072 ± 0.026	0.067 ± 0.031	0.059 ± 0.027	42.7 ± 9.6*
AM TNFα ng	0.425 ± 0.153	0.238 ± 0.081	0.353 ± 0.103	0.576 ± 0.393	4.01 ± 1.2*
Plasma TNFα[a]	0.005 ± 0.004	0.006 ± 0.005	0.007 ± 0.007	0.004 ± 0.003	0.006 ± 0.005

[a] Plasma TNFα levels are expressed as ng/ml. An asterisk denotes the responses differ from PBS treated rats ($p < 0.05$).

TNFα-B: 5′-GGAGGTTGACTTTCTCCTGGTATGAGA-3′. Results are expressed as pg iNOS and TNFα mRNA/ng cDNA.

Measurement of iNOS enzyme by Western blot. BAL fluid (0.5 ml) containing about 3-5 million cells were centrifuged at 1500 × g, 4 °C for 10 min. The pellets were then homogenized with 50 μl of homogenization buffer (20mM Tris-HCl, pH 7.5, 0.25M sucrose, 2mM EGTA, 2mM EDTA, 0.02%leupeptin, 1mM PMSF, 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 Beckman Tabletop Refrigerated TJ-9 centrifuge. The supernatants were stored at −20 °C. The protein concentration of the homogenates was determined by the BCA (bicinchoninic acid) method (17). Protein samples (50 μg) were separated on 10 % SDS-PAGE gels. Proteins were electrophoretically transferred to nitrocellulose using a Semi-Dry Transfer Cell (Bio-Rad Lab, Inc, Hercules, CA). The transfer buffer used was 48mM Tris-HCl and 39 mM glycine buffer (pH 9.2) containing 0.037% SDS and 20% methanol. After blocking nonspecific sites with blocking solution containing 5% (w/v) nonfat milk and 0.05% Tween 20 in phosphate buffered saline (PBS, 80 mM Na_2HPO_4, 20mM NaH_2PO_4,100 mM NaCl, pH 7.5) for 1 hr at room temperature, the nitrocellular membrane was incubated with polyclonal anti-rat iNOS antibody (Transduction Lab, Louisville, Kentucky) at a 1:5000 dilution in PBS containing 1% nonfat milk and 0.05% Tween 20) overnight at 4°C. After washing the membrane three times for 10 min each the membrane was incubated for 1 hr at room temperature with the horseradish peroxidase-linked secondary antibody (1:5,000 dilution in 1% nonfat milk and 0.05% Tween 20 in PBS), followed by washing in 0.05% Tween 20 in PBS for 10 min each of three times. The bound antibody on the membrane was detected by the enhanced chemiluminescence method according to the manufacturer's instructions (Amersham Corp, Arlington Height, IL). Exposure times of immunoblots to Hyperfilm were 1 min. The density of specific iNOS band was quantitated by using Foto Touch and SigmaGel densitometric analysis (Jandel Scientific, Sausalito, CA)

TNFα assays. TNFα was measured in lavage fluid and AM incubates with a commercially available ELISA kit (Genzyme Cambridge, MA) which utilizes a hamster anti-TNF monoclonal antibody and goat polyclonal anti-TNF antibody which recognize monomeric, dimeric, or trimeric TNFα as described previously in detail (13).

Statistical analysis of data. Each experiment contained 5-7 animals/treatment group. Data were analyzed with ANOVA for a randomized complete block or completely random sample design. Differences between and among means are analyzed with Dunnet's and Duncan's Tests. Biochemical data were analyzed with MANOVA and means compared with Newman-Kuehls test. $p<0.05$ was accepted for statistical differences between and among means.

RESULTS AND DISCUSSION

BALf cell counts, differential, and TNFα. We compared the ability of DB-cAMP and MT-ATP and LPS to up-regulate the inflammatory response by measurement of plasma and BAL fluid levels of TNFα and on the number and percentage of resident AM and PMN recruited into the alveolar space. Two hr. after i.t. administration of DB-cAMP (0.1 and 1.0 mg/kg) or MT-ATP the number and content of the cells in the BAL fluid did not differ from the PBS control (Table I). Moreover, these autocoids did not significantly up-regulate TNFα mRNA or protein in AM, BAL fluid or plasma (Table I).

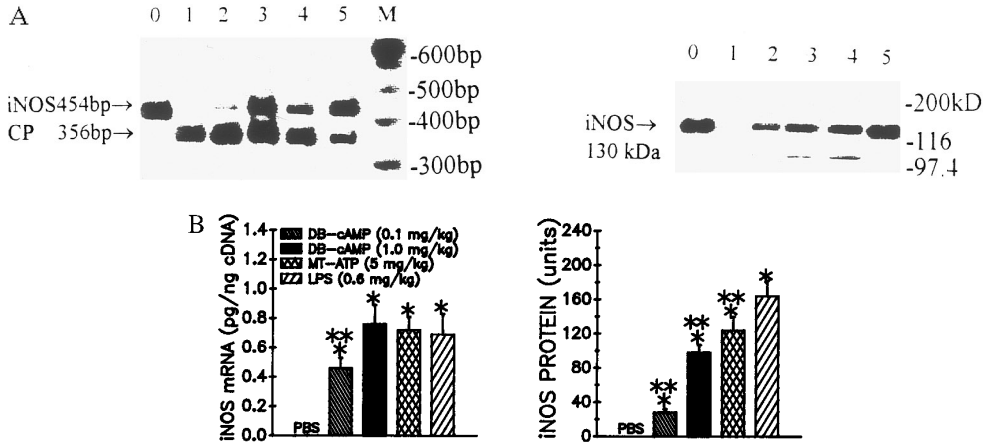

FIG. 1. Left Panel. Competitor equalized RT-PCR of iNOS mRNA in AM obtained from BAL fluid two hr. after treatment *in vivo* with PBS, DB-cAMP, MT-ATP or LPS. Lane 0 is the positive control for iNOS mRNA. Lane 1 is PBS, Lane 2 and 3 are DB-cAMP (0.1 and 1.0 mg/kg, i.t.), Lane 4 is MT-ATP (5 mg/kg, i.t.), Lane 5 is LPS (0.6 mg/kg, i.t.), Lane 6 is the kilobase marker. CP is the competitor DNA. The ratio of mRNA to cDNA corrected for background represents the change in iNOS expressed as pg mRNA/ng cDNA. Right Panel. Western blot of iNOS protein in AM obtained from BAL fluid two hr. after treatment of rats *in vivo* with i.t. PBS, DB-cAMP, MT-ATP or LPS. Lane 0 is the positive control for iNOS mRNA. Lane 1 is PBS, Lane 2 and 3 are DB-cAMP (0.1 and 1.0 mg/kg, i.t.), Lane 4 is MT-ATP (5 mg/kg, i.t.), Lane 5 is LPS (0.6 mg/kg, i.t.). The bottom is the mean and SE from 5 to 7 experiments per treatment group, each experiment reflecting an individual rat. An asterisk denotes the mean differs from PBS ($p<0.05$). A double asterisk indicates the mean and SE differ from LPS ($p<0.05$).

In contrast to DB-cAMP and MT-ATP, LPS increased the number of cells in the BAL fluid (Table I). The increased cells were PMN recruited into the alveolar space so that the percentage of AM in the BAL fluid decreased from 99.3 % to 64.3% of the total cell number (Table I). Further evidence for LPS-induced activation of the inflammatory response was presented by the two hr BAL fluid and AM incubate content of TNFα protein (Table I). This is the time at which TNFα secretion is maximum in response to LPS (12-16) and other cytokines after their i.t. administration. Finally, intrapulmonary generated TNFα remained compartmentalized within the lung since plasma levels were virtually undetectable (Table I). Thus DB-cAMP and MT-ATP, in contrast to LPS, do not provoke the inflammatory response when given via i.t. installation into the rat lung, *in vivo.*

Effect of LPS, DB-cAMP, and MT-ATP on iNOS mRNA, protein, and RNI. Inducible NOS mRNA and protein may be undetectable in cells treated with PBS because of the limits of sensitivity of the assay or because induction of gene transcription is a prerequisite for the control of iNOS enzyme activity. We have previously shown that competitor equalized RT-PCR quantitation of iNOS mRNA generates a standard curve with a correlation coefficient $>$ 0.94 and can be utilized with as little as 100,000 cells (13-16). To be certain that the levels of iNOS mRNA were simply not below the sensitivity of the assay to detect iNOS mRNA we utilized the protein and RNA from a million AM/sample, ten times the detectable limits of the assay. Inducible NOS mRNA was undetectable in AM obtained from PBS treated rats (FIG. 1). In contrast, two hours after i.t. administration of DB-cAMP and MT-ATP iNOS mRNA was increased in rat AM. The content of iNOS mRNA generated in AM obtained from rats treated DB-cAMP (1.0 mg/kg) or MT-ATP (5 mg/kg) was equal to or greater than that produced by LPS (FIG. 1). The mean data shows the consistency of the DB-cAMP and MT-ATP effects on iNOS mRNA (FIG. 1). It is unlikely that these autacoids increase iNOS

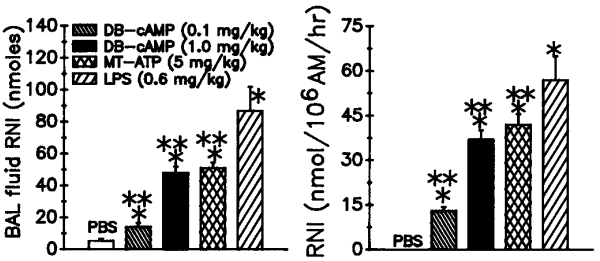

FIG. 2. Effect of intratracheal administration of PBS (0.,5 ml), DB-cAMP (0.1 and 1.0 mg/kg), MT-ATP (5 mg/kg) or LPS (0.6 mg/kg) on RNI levels in BAL fluid and AM. Vertical lines are SE.

mRNA by preventing its degradation by mRNA'ases since basal levels of iNOS mRNA were undetectable when obtained from PBS treated rats using ten times the number of AM in which iNOS mRNA can be detected. Moreover, the magnitude of induction of iNOS mRNA by these autocoids was equal to or greater than that produced by LPS which activates the transcription of iNOS mRNA and is devoid of inhibitory effects on iNOS degradation (1,2). Thus, DB-cAMP and MT-ATP induce iNOS mRNA, *in vivo*, independent of an inflammatory response, LPS-mediated priming of transcription of iNOS mRNA or release of endogenous TNFα.

In concordance with the levels of iNOS mRNA, treatment of rats with PBS failed to elicit any evidence of iNOS protein (FIG. 1). Moreover, DB-cAMP, MT-ATP and LPS induced iNOS protein in AM within 2 hr after their i.t. administration to the rat. However, in contrast to the equivalence of iNOS mRNA produced by the high dose of DB-cAMP, MT-ATP and LPS, translation of iNOS protein was significantly reduced in the AM obtained from the DB-cAMP and MT-ATP treated rats when compared to that found in the AM from LPS-treated rats (FIG. 1). The dimerization of the iNOS subunits is essential for post translational activation of iNOS activity. Among the factors promoting dimerization are arginine, heme, and tetrahydrobiopterin (BH4) (18,19). LPS increases arginine transport and the upregulation of BH4 in macrophages (20-23). DB-cAMP and MT-ATP do not increase the levels of arginine in AM (this laboratory, unpublished). Moreover, NO itself limits the dimerization of iNOS by diminishing the availability of heme iron to the enzyme (18). Speculatively, the observed differences in the levels of iNOS protein despite equivalent increases in iNOS mRNA by DB-cAMP (1 mg/kg), MT-ATP and LPS may result from limited post-translational dimerization of iNOS. This speculation is supported by the data obtained from BAL fluid and AM incubate concentrations of RNI. BAL fluid RNI was increased by DB-cAMP and MT-ATP two hr after i.t. administration to rats. The BAL fluid values were lower when obtained from the DB-cAMP and MT-ATP treated rats when compared to that of the LPS-treated rats. Similar results were obtained when the AM were incubated for an additional hr, *ex vivo* (FIG. 2). These data clearly indicate that DB-cAMP and MT-ATP, similar to LPS, generate functional iNOS *in vivo*. However, since cAMP increases the translation of NOS protein (1,2), the diminished iNOS protein and RNI may reflect a lesser degree of post-translational iNOS dimerization compared to LPS, because of the inability of the autocoids to upregulate arginine transport and induction of BH4 (22-23). Further studies are required to test this concept. Nevertheless, the data demonstrate for the first time that DB-cAMP and purine -2 receptor stimulation with MT-ATP can induce iNOS mRNA, protein and RNI in alveolar macrophages, in vivo, independent of LPS and without increases in TNFα.

Effect of diethyldithiocarbamate (DETC) on autacoid and LPS-induced iNOS mRNA and RNI. Induction of iNOS is primarily regulated at the level of transcription (1,2), and LPS up-

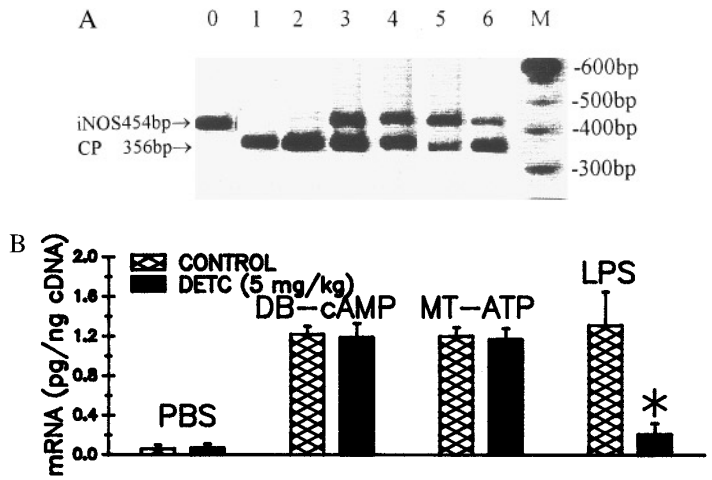

FIG. 3. Panel A. Effect of DETC (5 mg/kg, it, lanes 2, 4 and 6.) on AM iNOS mRNA induced by i.t. administration of PBS (lane 1 and 2), DB-cAMP (1 mg/kg, lanes 3 and 4) or LPS (0.6 mg/kg, lanes 5 and 6). DETC was given 15 min before drugs. The mean and SE from 5-7 experiments evaluating the effect of DETC on the iNOS mRNA responses to DB-cAMP (0.1 and 1 mg/kg), MT-ATP (5 mg/kg) or LPS (0.6 mg/kg) For details see legend to FIG. 1. * Differs from control in absence of DETC ($p<0.05$).

regulates iNOS mRNA by an NFκB dependent pathway (6). Therefore, we examined the role of NFkB in DB-cAMP and MT-ATP mediated upregulation of iNOS mRNA. Pretreatment of rats with DETC inhibited LPS-induced upregulation of iNOS mRNA but did not affect DB-cAMP or MT-ATP stimulated iNOS mRNA (FIG.3). Moreover, pretreatment of rats with DETC inhibited LPS-induced mediated upregulation of BAL fluid and AM incubate RNI without affecting that to DB-cAMP and MT-ATP. Addition of DETC to the incubates of AM obtained from rats treated with LPS in the absence of DETC did not affect the generation of RNI (FIG. 4).

DETC is an inhibitor of both NFκB and a scavenger of reactive oxygen intermediates, which have also been shown to induce the upregulation of NFκB (24). Activation of transcription factor NFκB seems to be an essential step for iNOS induction in most cells, in culture.

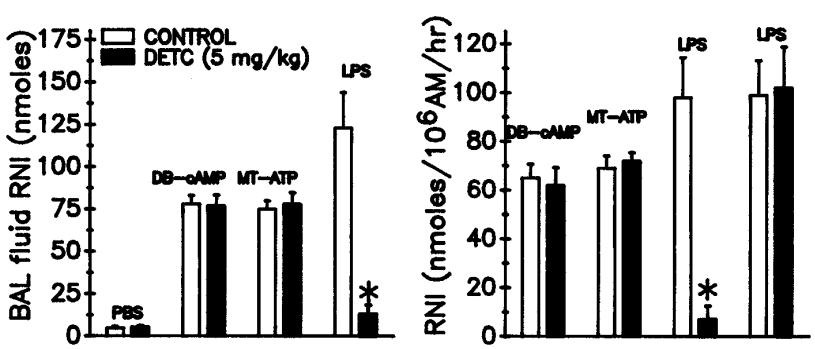

FIG. 4. Effect of DETC (5 mg/kg, i.t.) on BAL fluid (Left Panel) and AM incubate (Right Panel) concentrations of RNI determined by ozone chemiluminescence of acidified vanadium chloride reductants. Data are from 5-7 experiments. DETC was given 15 min before drugs except for second LPS bars in AM where it was added to activated AM incubates. An asterisk denotes the mean differs from control in absence of DETC ($p<0.05$).

However, different pathways resulting in the induction of this iNOS promoter may exist in different cells (2,6,25). Also, alternate promoters, such as AP-1 or AP-2, regulate gene transcription, especially, *in vivo* (24). The induction of iNOS mRNA and RNI production in isolated cells in culture requires approximately 8 hr (6,8,1,12). However, expression of iNOS mRNA and RNI is detected in AM and peripheral PMN within 15 min after LPS administration (13-16) and is present in significant levels within 2 hr after i.t. administration of DB-cAMP, MT-ATP and LPS (FIG. 1). This suggests the absence of a promoter or presence of an inhibitor *in vitro* which is not present *in vivo.* Since DETC inhibits LPS-induced transcription iNOS mRNA *in vivo* without affecting that to DB-cAMP or MT-ATP the transcription of iNOS mRNA in AM by these substances must utilize a pathway differing from that of LPS and independent of the transcription factor NFκB.

In conclusion, i.t. administration of LPS, DB-cAMP and MT-ATP rapidly upregulated iNOS mRNA, protein and RNI in AM, *in vivo.* Unlike LPS, these substances upregulated iNOS transcription without causing an inflammatory response. DB-cAMP and MT-ATP-mediated upregulation of iNOS transcription was refractory to inhibition by DETC, whereas LPS was inhibited by this inhibitor of NFκB and reactive oxygen intermediates. The data support the conclusions that purine-2- receptor and cyclic AMP stimulated, LPS and TNFα independent pathways exist for *in vivo* induction of iNOS transcription which also appear to be independent of NFκB. Thus, the finding of iNOS in patients with asthma (26) or coronary artery disease who consume medications that affect the cyclic AMP or purinergic systems may be iatrogenic rather than pathogenetic in origin.

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