cAMP and purinergic P$_{2y}$ receptors upregulate and enhance inducible NO synthase mRNA and protein in vivo

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Greenberg, Stan S., Xinfang Zhao, Ji-Fang Wang, Li Hua, and Jie Ouyang. cAMP and purinergic P$_{2y}$ receptors upregulate and enhance inducible NO synthase mRNA and protein in vivo. Am. J. Physiol. 273 (Lung Cell. Mol. Physiol. 17): L967–L979, 1997.—Adenosine 3',5'-cyclic monophosphate (cAMP) and purinergic P$_{2y}$ receptor agonists upregulate inducible nitric oxide (NO) synthase (iNOS) but inhibit Escherichia coli endotoxin lipopolysaccharide (LPS)- and cytokine-mediated upregulation of iNOS in cultured cells. We examined the effects of cAMP and P$_{2y}$ receptor agonists on the iNOS system in vivo. Intratracheal administration of dibutyryl-cAMP (DBcAMP, 0.1 and 1 mg/kg), a P$_{2y}$ receptor agonist (2-methylthioadenosine 5'-triphosphate (MeS-ATP), 5 mg/kg), or LPS (0.6 mg/kg) to rats 2 h before bronchoalveolar lavage (BAL) increased iNOS mRNA (competitor-equilibrated reverse transcription-polymerase chain reaction) and iNOS protein (Western blot) in rat alveolar macrophages compared with the effects of sterile phosphate-buffered saline (0.5 ml it). At equal levels of upregulation of iNOS mRNA, 1 LPS, but not DBcAMP or MeS-ATP, upregulated nuclear transcription factor-$\kappa$B (NF-$\kappa$B) and 2 iNOS protein and formation of NO were greater in alveolar macrophages from LPS- and MeS-ATP-treated rats than from DBcAMP-treated rats. Administration of DBcAMP or MeS-ATP 15 min before LPS did not inhibit LPS-induced alveolar macrophage-derived iNOS mRNA, iNOS protein, and NO. Diethylthiocarbamate (DTC, 5 mg/kg it) inhibited LPS-induced iNOS mRNA but did not affect upregulation of iNOS mRNA produced by the other agonists. We conclude that an LPS-dependent and -independent pathway of iNOS mRNA induction exists in vivo. The former is activated by LPS and most cytokines, is associated with upregulation of NF-$\kappa$B and inhibited by DTC, and elicits an inflammatory response. The latter, activated by DBcAMP and MeS-ATP, is not associated with upregulation of NF-$\kappa$B, inhibition by DTC, or activation of inflammation. The two systems are additive in vivo rather than antagonistic. Speculatively, if the LPS-independent iNOS pathway exists in humans, the iNOS in tissues from patients taking drugs affecting cAMP or P$_{2y}$ receptors may be iatrogenic rather than pathogenetic in origin.

transcription factors; tumor necrosis factor-$\alpha$; diethylthiocarbamate; adenosine 3',5'-cyclic monophosphate; nitric oxide; lipopolysaccharide

ALVEOLAR MACROPHAGES (AM) are the first line of defense against airborne-derived pathogens. Exposure of AM to pathogenic organisms or their lipopolysaccharide (LPS) membrane coat activates this phagocytic cell for production of cellular mediators that aid in the killing and/or phagocytosis of the foreign organism. Among these mediators are cytokines, arachidonic acid metabolites, and oxygen- and nitrogen-derived free radicals, including nitric oxide (NO) and peroxynitrite (20, 28, 31, 32, 44, 45, 48, 49). NO, a free radical, is the smallest known bioactive molecule produced by almost every cell in almost all species, including humans (17, 28). High concentrations of this free radical, such as the concentrations produced in AM by the inducible isozyme of NO synthase (iNOS), can destroy bacteria, parasites, and specific types of tumor cells (17, 20, 28, 31, 46). The binding of NO to iron molecules can inhibit the enzymatic activity of these pathogens as well as their host cells (17, 28). Moreover, by inhibiting the iron-responsive elements on iNOS mRNA or iNOS protein and by posttranslational mechanisms involving the ADP ribosylation of proteins, NO can prevent the formation of new enzymes or modify mRNA and cellular proteins and, thereby, their functions (7, 10, 17, 28). In view of the diverse nature and multiple sites of action of NO within the cell, it is important to define the factors that modulate the regulation of iNOS in vivo.

iNOS (EC 1.14.13.39) is an isoform of the NOS family of enzymes. It is usually absent in resting cells and can be induced by cytokines and bacterial cell wall products such as the interleukins, tumor necrosis factor-$\alpha$ (TNF-$\alpha$), and Escherichia coli endotoxin LPS (4, 17, 28). However, recent studies suggest that a rapid increase and decrease in iNOS mRNA may occur through the action of a cycloheximide-inhibitable protein (16), which acts by prolonging the lifetime of iNOS mRNA (23). These proteins may be promoters or enhancers of iNOS transcription. Nevertheless, the question of whether some cells can constitutively express iNOS remains unanswered. However, the induction of iNOS appears to be primarily regulated at the level of gene transcription by cytokines and LPS as a result of their ability to activate protein and tyrosine kinases (7, 13, 16, 17, 19, 24, 25, 28) and promoters and nuclear transcription factors such as nuclear factor-$\kappa$B (NF-$\kappa$B) and inducible regulatory factor type 1 (IRF-1) (3, 7, 29, 47, 48). Glucocorticoids, which inhibit LPS-induced gene expression for iNOS, appear to inhibit gene expression by interfering with an adenosine 3',5'-cyclic monophosphate (cAMP)-responsive enhancer (CREB) of transcription (1). Recent studies, using cultured cells and cell lines, also demonstrated that many of the cell-signaling pathways involved in the expression of iNOS appear to be modulated by purinergic P$_{2y}$ receptors and by cAMP (2, 8, 12, 14, 17, 24, 26, 30, 34, 37–39, 44).

Compounds that stimulate P$_{2y}$ receptors or upregulate the cAMP system act synergistically with LPS to upregulate iNOS protein in the RAW 264.7 murine macrophage cell line (43), in cultured rat vascular smooth muscle (14, 24) and mesangial cells (38); in cardiac myocytes in cell culture (39) by enhancement of translation or by inhibition of the degradation of iNOS mRNA and/or protein. Moreover, prolonged incubation
of murine fibroblasts with cAMP in cell culture induces iNOS mRNA and iNOS protein (6) while augmenting cytokine-stimulated NO synthesis in cultured cardiac myocytes (26) and vascular smooth muscle cells (30). However, cAMP and a P2y receptor agonist [2-methylthioadenosine 5’-triphosphate (MeS-ATP)] did not directly affect iNOS but suppressed LPS-mediated upregulation of iNOS mRNA and the production of NO in isolated astrocytes (37) and in cultured murine macrophages (8, 12). These data suggest that a cytokine-independent pathway exists for cAMP and P2y receptor agonists to upregulate or downregulate the iNOS system by inhibition or facilitation of the degradation or stability of iNOS mRNA in vitro (12, 29, 39). Nevertheless, the role of these autacoids as modulators of iNOS in vivo remains undefined. Thus we examined the effects of upregulation of the cAMP system and purinergic P2y receptor stimulation on iNOS mRNA, iNOS protein, and NO production of rat AM in vivo and the interaction of these agonists with LPS on the NOS system. We also tested the ability of these agonists to upregulate NF-κB.

MATERIALS AND METHODS

Direct effects of autacoids. Conventional male Sprague-Dawley rats (Hilltop Farms, Scottsdale, PA), weighing 225–250 g, were housed at the vivarium of the Louisiana State University Medical Center at New Orleans under a 12:12-h 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 given 0.5 ml of sterile phosphate-buffered saline (PBS) or test compounds dissolved in 0.5 ml of PBS by intratracheal (i.t.) gavage. The test compounds used were E. coli endotoxin (LPS serotype 026:B6, 0.6 mg/kg; Difco, Detroit, MI), dibutyryl-cAMP (DBcAMP, 0.1 or 1 mg/kg; Research Biochemicals, Cleveland, OH), MeS-ATP (5 mg/kg; Research Biochemicals), albuterol (0.5 mg/kg; University Hospital, New Orleans, LA), or isoproterenol (0.2 µg/kg; Sigma Chemical, St. Louis, MO). The neck wounds were closed, and the animals were allowed to recover. Two hours after intratracheal 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 TNF-α and NO2/NO3 [reactive nitrogen intermediates (RNI)]. The heart and lungs were removed, and the lung was subjected to bronchoalveolar lavage (BAL) with 30 ml of PBS. The BAL fluid was analyzed by centrifugation at 5,000 g for 15 min at 4°C, and the incubate was assayed for RNI with ozone chemiluminescence or TNF-α with the WEHI assay or with an enzyme-linked immunosorbent assay (31, 46).

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 NO. Determination of the NO formed from RNI was made by measuring the specific chemiluminescence resulting from the reaction of NO with machine-generated ozone using an NO-NOx analyzer (model 821, Dasibi Environmental, Glendale, CA). Conversion of standard solutions of nitrite to NO was >99%, whereas conversion of nitrate to NO was 94–96% compared with calibrated standards of NO gas (20, 31, 46). Conversion of 5-nitroso-N-acetylpenicillamine to NO was also >99%.

Assay of mRNA for iNOS and TNF-α. Transcripts for iNOS and TNF-α were measured by cDNA-quantified reverse transcription-polymerase chain reaction (cERT-PCR) in lavaged cells, as previously described (20, 31, 45). Briefly, the total RNA of the AM was isolated using TRIzol reagent (GIBCO, Gaithersburg, MD). Total cDNA was obtained by reverse transcription of total RNA and labeled with [32P]dCTP. Total cDNA (10 ng) was amplified together with competitor (1 pg) by using the same specific iNOS or TNF-α primers and [32P]dCTP. Primer sequences for iNOS and TNF-α were as follows: 5'-AATGCGCAATCAGGTGGCCCATC3' (iNOS-A) and 5'-GCTGTGTCACAGAAGTCGAC3' (iNOS-B); 5'-AAGTCTTTGAAATGGGCTCTCCATC3' (TNF-α-A) and 5'-GGAGTTGACTTTTTCTCAGTGA-
GA-3′ (TNF-α-B). Amounts of iNOS and TNF-α cDNA were determined by phosphorimage scan and quantitation of the smear and signal bands normalized to the competitor. The total cDNA formed (in ng) equaled the gray value of the smear divided by the gray value of the standard (× 330). The average molecular weight of dNTP (dATP, dGTP) was 330 is the average molecular weight of dNTP (dATP, dGTP, dCTP, dTTP) and [dCTP] is dCTP concentration. The amount of iNOS or TNF-α mRNA (in pg/µg cDNA) equaled ([iNOS or TNF-α] g gray value – background) ÷ (competitor g gray value – background) × (1 pg competitor/10 ng total cDNA). The results were expressed as picograms of iNOS or TNF-α mRNA per nanogram of cDNA and were compared with standard curves plotted by addition of known amounts of iNOS or TNF-α mRNA standards and their competitors to cell samples (n = 8) devoid of any measurable iNOS or TNF-α mRNA.

Measurement of iNOS enzyme by Western blot. BAL fluids (0.5 ml) containing 3–5 × 10⁵ cells were centrifuged at 1,500 g for 4°C in a min. The pellets were then homogenized with 50 µl of homogenization buffer [20 mM tris(hydroxymethyl)ammonium (Tris)-HCl, pH 7.5, 0.25 M sucrose, 2 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 2 mM EDTA, 0.02% leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 0.1% Triton X-100]. The homogenates were incubated for 1 h at 4°C and centrifuged at 3,000 revolutions/min for 30 min at 4°C in a tabletop refrigerated centrifuge (model TJ-9, Beckman Instruments, Fullerton, CA). The supernatants were stored at −20°C. The concentration of protein in the homogenates was assessed by the bicinchoninic acid method (18). Protein samples (50 µg) were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. Proteins were electrophoretically transferred to nitrocellulose using a semidry transfer cell (Bio-Rad, Hercules, CA). The transfer buffer was 48 mM Tris·HCl and 99–99.8% of which were also AM (Tables 1 and 2). Statistical analyses of data. Each experiment was replicated with 6–22 rats/group. Data were analyzed with analysis of variance for a randomized complete block or completely random sample design. Differences between and among means were analyzed with Dunnett’s and Duncan’s tests. Biochemical data were analyzed with multivariate analysis of variance, and means were compared with Newman-Keuls test. P ≤ 0.05 was accepted for statistical significance of mean differences.

RESULTS

BAL fluid cell counts and differential. The control rats given PBS had a total BAL fluid cell content of 15.2 ± 1.3 (n = 22) to 18.9 ± 0.8 × 10⁶ (SE) cells (n = 11), 99–99.8% of which were also AM (Tables 1 and 2). This did not differ from the cell type and distribution obtained from the BAL fluid of untreated rats given anesthetic before lavage (Table 1). Treatment of rats with LPS (0.6 mg/kg it) increased the total number of cells by increasing the influx of PMN into the lung (Table 1). In contrast, pretreatment of rats with DBcAMP, MeS-ATP, isoproterenol, and albuterol did not significantly affect the number of AM or PMN recovered from the BAL fluid (Table 1). Pretreatment of rats with DETC inhibited LPS-stimulated AM as well as PMN recruitment into the alveolar space, thereby decreasing the total cell number without affecting the relative distribution of AM and PMN (Table 2). Although DETC slightly enhanced the accumulation of neutrophils within the lung, the effect was minor and did not override the lack of effect of DBcAMP, MeS-ATP, or isoproterenol on the AM or PMN (Table 2). Administration of DBcAMP or MeS-ATP with LPS slightly decreased the total number of AM in the BAL fluid, thus increasing the percentage but not the number of LPS-stimulated PMN in the alveolar space (Table 1).
Table 1. Effect of DBcAMP, MeS-ATP, and LPS on white cell distribution in lung

<table>
<thead>
<tr>
<th></th>
<th>Cells, (\times 10^6)</th>
<th>AM</th>
<th>PMN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>15.9 ± 1.4</td>
<td>15.8 ± 1.4</td>
<td>99 ± 0.8</td>
</tr>
<tr>
<td>PBS</td>
<td>15.2 ± 1.3</td>
<td>15.2 ± 1.3</td>
<td>99 ± 0.7</td>
</tr>
<tr>
<td>LPS</td>
<td>23.2 ± 2.1*</td>
<td>15.0 ± 1.0</td>
<td>67 ± 5.2*</td>
</tr>
<tr>
<td>DBcAMP</td>
<td>0.1 mg/kg</td>
<td>17.2 ± 0.7</td>
<td>17.0 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>1 mg/kg</td>
<td>16.5 ± 0.9</td>
<td>16.4 ± 0.9</td>
</tr>
<tr>
<td>MeS-ATP</td>
<td>16.6 ± 1.1</td>
<td>16.5 ± 1.1</td>
<td>99 ± 0.4</td>
</tr>
<tr>
<td>ISO</td>
<td>16.9 ± 1.0</td>
<td>16.8 ± 1.0</td>
<td>100 ± 0.1</td>
</tr>
<tr>
<td>Albuterol</td>
<td>15.5 ± 0.4</td>
<td>15.3 ± 0.4</td>
<td>99 ± 0.3</td>
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</table>

Values are means ± SE for 10–22 rats. Each dose of drug was dissolved in 4.5 ml of phosphate-buffered saline (PBS), or 4.5 ml of PBS were administered by intratracheal injection, and lungs were lavaged 2 h later. Total count and cell differential were performed as described in MATERIALS and METHODS. DBcAMP, dibutyryl cAMP 3',5'-cyclic monophosphate (0.1 and 1 mg/kg, as indicated); MeS-ATP, 2-methylthioadenosine 5'-triphosphate (5 mg/kg); ISO, isoproterenol (0.02 µg/kg); LPS, Escherichia coli endotoxin (lipopolysaccharide, 0.6 mg/kg); AM, alveolar macrophages; PMN, polymorphonuclear leukocytes. *Significantly different from PBS control.

Conversely, pretreatment of rats given the combination of autacoid mimetic and LPS with DETC decreased the total number of cells in the BAL fluid, with a greater effect on PMN. Thus the relative distribution of AM and PMN returned to that seen with DETC and LPS alone (Table 2).

Direct effects of LPS and autacoids on the iNOS system of rat AM. Treatment of rats with LPS, DBcAMP, and MeS-ATP upregulated iNOS mRNA in AM within 2 h after intratracheal administration. The content of iNOS mRNA generated in AM from rats treated with DBcAMP (1.0 mg/kg) or MeS-ATP was equal to or greater than that produced by LPS (Fig. 1). LPS, DBcAMP, and MeS-ATP increased the levels of iNOS protein within AM 2 h after intratracheal administration to rats. The levels of iNOS protein produced by the low and high dose of DBcAMP for equivalent levels of iNOS mRNA produced in the AM were lower than those produced in the AM from LPS-treated rats (Fig. 1). PBS, isoproterenol, and albuterol did not induce iNOS mRNA or iNOS protein (Fig. 1). Means ± SE from 5–22 individual experiments are summarized in Fig. 2.

RNI production. A basal level of RNI existed in the BAL fluid of rats treated with PBS (Fig. 3, top). The RNI generated by AM from PBS-treated rats incubated ex vivo for 1 h did not differ from that of the buffer in the absence of cells (Fig. 3, bottom). Pretreatment of rats with LPS, DBcAMP, or MeS-ATP increased RNI levels in BAL fluid and the ex vivo incubates of AM (Fig. 3, bottom). However, the increase in RNI induced by the autacoid mimetics was less than that in the BAL fluid.

Table 2. Effect of DETC on DBcAMP-, MeS-ATP-, and LPS-induced changes in white blood cells

<table>
<thead>
<tr>
<th></th>
<th>Cells, (\times 10^6)</th>
<th>AM</th>
<th>PMN</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>18.9 ± 0.6</td>
<td>18.7 ± 0.6</td>
<td>100 ± 0.4</td>
</tr>
<tr>
<td>DETC + PBS</td>
<td>15.9 ± 0.4*</td>
<td>15.5 ± 0.4*</td>
<td>98 ± 1.2</td>
</tr>
<tr>
<td>PBS + LPS</td>
<td>31.7 ± 2.2*</td>
<td>22.5 ± 2.3</td>
<td>71 ± 6.1*</td>
</tr>
<tr>
<td>DETC + LPS</td>
<td>19.5 ± 0.8†</td>
<td>13.3 ± 0.8†</td>
<td>67 ± 0.9*</td>
</tr>
<tr>
<td>PBS + DBcAMP (0.1 mg/kg)</td>
<td>18.9 ± 2.5</td>
<td>18.7 ± 0.6</td>
<td>99 ± 0.4</td>
</tr>
<tr>
<td>DETC + DBcAMP (0.1 mg/kg)</td>
<td>18.8 ± 2.5</td>
<td>16.9 ± 2.2</td>
<td>90 ± 1.7*</td>
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<tr>
<td>PBS + DBcAMP (1 mg/kg)</td>
<td>22.4 ± 2.1</td>
<td>22.1 ± 2.1</td>
<td>99 ± 0.4</td>
</tr>
<tr>
<td>DETC + DBcAMP (1 mg/kg)</td>
<td>19.8 ± 0.7</td>
<td>18.2 ± 0.9</td>
<td>92 ± 2.6</td>
</tr>
<tr>
<td>PBS + MeS-ATP</td>
<td>20.2 ± 2.4</td>
<td>19.7 ± 1.5</td>
<td>99 ± 0.4</td>
</tr>
<tr>
<td>DETC + MeS-ATP</td>
<td>16.9 ± 2.7</td>
<td>18.6 ± 1.2</td>
<td>99 ± 0.6</td>
</tr>
<tr>
<td>PBS + ISO</td>
<td>23.4 ± 3.9</td>
<td>22.6 ± 3.1</td>
<td>97 ± 2.4</td>
</tr>
<tr>
<td>DETC + ISO</td>
<td>20.0 ± 2.6</td>
<td>19.6 ± 1.5</td>
<td>98 ± 1.6</td>
</tr>
<tr>
<td>PBS pretreated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DBcAMP (0.1 mg/kg) + LPS</td>
<td>27.7 ± 3.3*</td>
<td>16.2 ± 1.3*</td>
<td>60 ± 2.4*</td>
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<tr>
<td>DBcAMP (1 mg/kg) + LPS</td>
<td>28.4 ± 4.6*</td>
<td>17.2 ± 2.3*</td>
<td>62 ± 2.5*</td>
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<tr>
<td>MeS-ATP + LPS</td>
<td>32.6 ± 7.0*</td>
<td>18.8 ± 4.5</td>
<td>56 ± 2.1*</td>
</tr>
<tr>
<td>DETC treated</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>+ DBcAMP (0.1 mg/kg) + LPS</td>
<td>19.2 ± 1.3*</td>
<td>13.2 ± 0.7†</td>
<td>67 ± 0.9†</td>
</tr>
<tr>
<td>+ DBcAMP (1 mg/kg) + LPS</td>
<td>18.6 ± 1.2*</td>
<td>12.7 ± 0.5†</td>
<td>67 ± 0.9†</td>
</tr>
<tr>
<td>+ MeS-ATP + LPS</td>
<td>19.1 ± 1.3*</td>
<td>13.1 ± 0.8†</td>
<td>67 ± 0.9†</td>
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</table>

Values are means ± SE for 6–22 rats. Drugs were dissolved in 4.5 ml PBS, or 4.5 ml of PBS were administered by intratracheal injection, and lungs were lavaged 2 h later. In animals pretreated with PBS, DBcAMP, or MeS-ATP before administration of LPS, autacoid mimetics were given 15 min before LPS, and lungs were subjected to lavage 2 h after administration of LPS. PBS or diethylthiocarbamate (DETC) (5 mg/kg) was given 30 min before injection of LPS. Total count and cell differential were performed as described in MATERIALS and METHODS. See Table 1 for definition of other abbreviations and other drug doses. *Significantly different from PBS control or PBS-pretreated control. †Significantly different from PBS control and from same treatment without DETC.
and incubates of AM from LPS-treated rats. The two β-adrenoceptor agonists that did not induce mRNA transcription or iNOS protein did not affect RNI levels in the BAL fluid or in the ex vivo incubates of AM (Fig. 3).

Effect of LPS and autacoids on TNF-α. Low background levels of TNF-α mRNA were present in freshly isolated AM from rats treated with PBS, DBcAMP, MeS-ATP, albuterol, or isoproterenol and did not differ among the treatment groups (P > 0.67). TNF-α mRNA and immunoreactive and biologically active TNF-α protein were only increased when measured in AM from LPS-treated rats (Fig. 4).

Effect of DBcAMP and MeS-ATP on LPS-stimulated iNOS mRNA, iNOS protein, and RNI. The contents of LPS-induced iNOS mRNA and iNOS protein in AM from rats pretreated with the autacoid mimetics were greater than those produced by administration of the autacoid mimic or LPS individually to rats (Fig. 5). The magnitude of DBcAMP (1 mg/kg)- or MeS-ATP-stimulated enhancement of LPS-mediated upregulation of iNOS mRNA and iNOS protein in rat AM was additive rather than synergistic with that of LPS (Figs. 5 and 6). However, DBcAMP (0.1 mg/kg)-induced enhancement of LPS-mediated upregulation of iNOS mRNA and iNOS protein was synergistic with LPS in AM from rats pretreated with this low dose of DBcAMP. LPS-induced increases of RNI in BAL fluid and ex vivo incubates of AM were also enhanced in an additive and synergistic manner in rats pretreated with DBcAMP or MeS-ATP (Fig. 7). Pretreatment of rats with PBS, isoproterenol, or albuterol did not affect LPS-mediated upregulation of iNOS mRNA, iNOS protein, or RNI (Figs. 5–7).

Effect of DBcAMP and MeS-ATP on LPS-stimulated TNF-α. Pretreatment of rats with DBcAMP or MeS-ATP did not affect LPS-induced increases in AM TNF-α.

Fig. 1. Representative gels of inducible NO synthase (iNOS) mRNA and iNOS protein in alveolar macrophages (AM) from rats treated with phosphate-buffered saline (PBS), autacoid mimetics, or β2-adrenoceptor agonists. AM were obtained from bronchoalveolar lavage (BAL) fluid, and total mRNA was isolated and subjected to cDNA-quantitative reverse transcription-polymerase chain reaction (cERT-PCR) or iNOS protein was isolated using Western blot analyses and a polyclonal antibody to iNOS. Gel density of iNOS signal was determined with computerized gel densitometry. Top: cERT-PCR of iNOS mRNA in AM from BAL fluid 2h after intratracheal treatment in vivo with PBS (0.5 ml; lane 1), lipopolysaccharide (0.6 mg/kg; lane 2), dibutyryl-cAMP (0.1 and 1 mg/kg; lanes 3 and 4), 2-methylthioadenosine 5'-triphosphate (5 mg/kg; lane 5), isoproterenol (0.2 µg/kg; lane 6), or albuterol (0.5 mg/kg; lane 7). Lane 0, positive control for iNOS mRNA (top) or iNOS protein (bottom); Lane M, kilobase marker for RNA or molecular mass marker for iNOS protein. CP, competitor DNA (cDNA). Ratio of mRNA to cDNA corrected for background represents differences in iNOS mRNA. The less dense the cDNA, the more iNOS mRNA is present. Bottom: the less dense the band, the less iNOS protein is present. For calculations see MATERIALS AND METHODS.

Fig. 2. Effect of PBS, autacoid mimetics, and lipopolysaccharide (LPS) on rat AM iNOS mRNA (top) and iNOS protein (bottom). Top: ratio of iNOS mRNA to competitor DNA corrected for background (ordinate) represents iNOS mRNA expressed as pg mRNA/ng cDNA. Bottom: Western blot gel density of iNOS protein in 50 mg of protein for AM from BAL fluid 2h after intratracheal treatment of rats in vivo with PBS, LPS, dibutyryl-cAMP (DBcAMP), 2-methylthioadenosine 5'-triphosphate (MeS-ATP), isoproterenol (Iso), or albuterol (Alb), as described in Fig. 1 legend. Values are means ± SE for 5–22 rats/group. *Significantly different (P < 0.05) from PBS. †Significantly different (P < 0.05) from LPS.
mRNA (Fig. 8, top). However, each of these autacoids inhibited the release of biologically active and immunoreactive TNF-α in the BAL fluid and ex vivo incubates of AM (Fig. 8, top and bottom). Pretreatment of rats with PBS, isoproterenol, or albuterol did not affect LPS-mediated upregulation of TNF-α mRNA in AM or TNF-α protein levels in the BAL fluid and ex vivo incubates of AM (Fig. 8).

Effect of LPS, DBcAMP, and MeS-ATP on NF-κB. The pathway utilized by LPS and the autacoid mimetics was explored on a limited basis. Nuclear extracts of AM from rats treated with PBS, LPS, DBcAMP (1 mg/kg), and MeS-ATP were studied at 2 h, since preliminary studies showed that the peak in vivo change in NF-κB occurred at this time in AM (unpublished observations). LPS increased NF-κB, as determined by EMSA (Fig. 8) and verified by the supershift assay (data not shown). In contrast, concentrations of DBcAMP and MeS-ATP that increased iNOS mRNA did not increase NF-κB compared with PBS-treated rats (Fig. 9), which also did not express iNOS mRNA (Fig. 1).

Effects of DETC on LPS, DBcAMP, and MeS-ATP. Pretreatment of rats with DETC inhibited LPS-mediated upregulation of iNOS mRNA, iNOS protein, and RNI levels in the BAL fluid and ex vivo incubates of AM but did not affect DBcAMP- and MeS-ATP-mediated increases in these parameters (Figs. 10 and 11). Moreover, administration of DETC to rats pretreated with DBcAMP or MeS-ATP before intratracheal administration of LPS abolished the component of the enhanced iNOS mRNA and iNOS protein attributable...
to LPS (Figs. 10 and 12). The residual response to the coadministration of these autacoid mimetics and LPS remaining in DETC-pretreated rats equaled that produced by DBcAMP or MeS-ATP alone (Figs. 10 and 12). Pretreatment of rats with DETC did not affect the ability of LPS to upregulate TNF-α mRNA, immunoreactive or biologically active (data not shown) TNF-α protein, or DBcAMP or MeS-ATP to suppress LPS-mediated upregulation of TNF-α biologically active (data not shown) and immunoreactive (Fig. 13) TNF-α protein in BAL fluid and ex vivo incubates from AM.

**DISCUSSION**

New findings of the study. This is the first study to demonstrate that in vivo administration of low doses of DBcAMP and the P2y receptor partial agonist MeS-ATP directly stimulated transcription of iNOS mRNA and translation of the mRNA to active iNOS protein, which in turn produced RNI in the BAL fluid and in the 1-h ex vivo incubates of the recovered AM. Furthermore, intratracheal administration of LPS enhanced the ability of LPS to upregulate iNOS mRNA and iNOS protein in the freshly isolated AM and the resultant RNI in the BAL fluid and 1-h ex vivo incubates of the recovered AM. In contrast to LPS, the autacoid mimetics upregulated the iNOS system without eliciting activation of the inflammatory process, since TNF-α was not increased and recruitment of PMN into the alveolar space did not occur. These data suggest that local regulation of iNOS transcription in AM in the rat may result from the activity of endogenous cAMP and P2y receptor stimulation of the AM in the mouse and rat (12, 34, 44) and potentially different tissues in several species (2, 14, 17, 24, 26, 28–30, 37–39). It may be argued that if this postulate were correct, the β-adrenoceptor agonists should have increased iNOS mRNA and iNOS protein, whereas they were devoid of effects on the iNOS system in rat AM in vivo. However, isoproterenol and albuterol were used as negative controls to test for nonspecific irritation of the lung and thereby activation of the AM because rat AM are essentially devoid of β-adrenoceptors. Furthermore, LPS selectively increased NF-κB and DETC, an inhibitor of NF-κB, and free radical-mediated activation of NF-κB (15, 29, 36, 47) selectively attenuated LPS-mediated upregulation of iNOS mRNA and iNOS protein from rat AM 2 h after intratracheal administration of LPS. Top: ratio of iNOS mRNA to competitor DNA corrected for background (ordinate). Bottom: Western blot gel density of iNOS protein in AM obtained from BAL fluid 2 h after treatment of rats in vivo with intratracheal PBS. Rats were pretreated with PBS, DBcAMP, MeS-ATP, or Iso as described in Fig. 5 legend. Values are means ± SE for 5–17 rats/group. *Significantly different (P < 0.05) from LPS alone. **Significantly different (P < 0.05) from LPS + autacoid mimetic (P < 0.05).

Fig. 6. Autacoid-induced enhancement of LPS-mediated upregulation of iNOS mRNA and iNOS protein from rat AM 2 h after intratracheal administration of LPS. Values are means ± SE for 5–17 rats/group. *Significantly different (P < 0.05) from LPS alone. **Significantly different (P < 0.05) from LPS + autacoid mimetic (P < 0.05).

Fig. 7. Autacoid-induced enhancement of LPS-mediated upregulation of RNI in BAL fluid (top) and ex vivo incubates of AM (bottom). Values are means ± SE for 5–17 rats/group. *Significantly different (P < 0.05) from LPS alone. **Significantly different (P < 0.05) from LPS + autacoid mimetic (P < 0.05).
of transcription of iNOS mRNA. Thus the data also support the postulate that the cell-signaling pathway utilized by the autacoid mimetics differed from that utilized by LPS. This finding is in agreement with that which demonstrated that dexamethasone differentially inhibited cAMP- but not interleukin-1β-induced upregulation of iNOS mRNA in renal mesangial cells (33). Moreover, posttranscriptional and translation mechanisms may be differentially modulated by the autacoid and LPS pathways. This conclusion is based on the finding that disparities existed between the amount of iNOS protein and generated NO produced in the LPS-, MeS-ATP-, and DBcAMP-stimulated AM that contained equal levels of iNOS mRNA.

For equivalent amounts of iNOS mRNA produced by the high dose of DBcAMP, MeS-ATP, and LPS, the amounts of iNOS protein and RNI generated by AM from the rats treated with DBcAMP were slightly but significantly lower than the amounts produced by LPS. Moreover, the high dose of DBcAMP interacted with LPS in an additive manner, whereas the low dose of DBcAMP interacted with LPS in a synergistic fashion. These findings, in agreement with others using cultured cells (12, 29, 30, 33), further support the conclusion that the autacoid mimetics and LPS upregulate the iNOS system via distinct cell-signaling pathways (12, 29).

Effects of DETC. LPS-induced upregulation of the iNOS system is dependent on activation of a tyrosine kinase (13, 17, 28) or an isozyme of protein kinase C (17, 25, 28) and subsequent activation of NF-κB (this study; 17, 28, 29, 47) that is inhibited by DETC and pyrrolidine dithiocarbamate (15, 36, 43). We also demonstrated that in vivo administration of the protein kinase C inhibitors staurosporine and chelerythrine to rats did not affect DBcAMP- and MeS-ATP-induced upregulation of iNOS mRNA (21). This study demonstrated that pretreatment of rats with LPS, but not with DBcAMP or MeS-ATP, selectively upregulated NF-κB- and DETC-inhibited LPS-mediated transcription of iNOS mRNA and iNOS protein in AM in vivo. The cell-signaling pathways utilized by the autacoids and LPS must differ, since a dose of DETC that inhibited LPS-mediated upregulation of iNOS mRNA and iNOS protein failed to affect the iNOS mRNA or the iNOS protein induced by the autacoids, which did not upregulate NF-κB. Moreover, in experiments in which LPS was administered with the autacoid mimetics, the iNOS mRNA, iNOS protein, and RNI remaining after the pretreatment of rats with DETC equaled that produced by the autacoid mimetics alone. These findings support the conclusion that the pathway utilized by DBcAMP and MeS-ATP to upregulate transcription of iNOS in rat AM in vivo differed from that utilized by LPS and appears to be independent of NF-κB.

**Fig. 8.** Autacoid-induced inhibition of LPS-mediated upregulation of biologically active and immunoreactive TNF-α in BAL fluid (top) and 1-h ex vivo incubates of rat AM (bottom). Values are means ± SE for 5–17 rats/group. *Significantly different (P < 0.05) from LPS alone. †Significantly different (P < 0.05) from LPS + autacoid mimic. For details see Fig. 5 legend.

**Fig. 9.** Effects of intratracheal administration of PBS (0.5 ml), LPS (0.6 mg/kg), DBcAMP (1 mg/kg), and MeS-ATP (5 mg/kg) on nuclear factor-κB (NF-κB) DNA binding activity in nuclear extracts of AM. Rats were killed 2 h after drug administration. AM were isolated from BAL fluid, and nuclear extracts were isolated. Nuclear extracts (5 μg/well) were analyzed by electrophoretic mobility shift assay for NF-κB DNA binding. Gel is representative of 4 separate experiments.
Mechanism of action of DBcAMP and MeS-ATP. It is possible that these autacoids increased endogenous constitutive iNOS mRNA by preventing its degradation by messenger ribonucleases, as it does in cardiac myocytes in cell culture (39). iNOS mRNA was undetectable in AM from PBS-treated rats. Although this may have resulted from the limits of sensitivity of the assay (10–5 pg/ng cDNA), this is likely, since we used 2–4 × 10⁶ AM/ml to measure iNOS mRNA and protein, 10 times the number of cells in which iNOS mRNA and protein are detectable in AM from PBS-treated rats. Although this may have resulted from the limits of sensitivity of the assay (10–5 pg/ng cDNA), this is likely, since we used 2–4 × 10⁶ AM/ml to measure iNOS mRNA and protein, 10 times the number of cells in which iNOS mRNA and protein are detectable in AM from PBS-treated rats.

Fig. 10. A: representative cERT-PCR and Western blot gels (50 µg of applied protein) showing effects of PBS (0.5 ml it; lanes 1, 3, 5, 6, and 9) and diethyldithiocarbamate (DETC, 5 mg/kg it; lanes 2, 4, 7, 8, and 10) on rat AM iNOS mRNA and iNOS protein induced by intratracheal administration of PBS (lanes 1 and 2), LPS (0.6 mg/kg; lanes 3 and 4), DBcAMP (0.1 mg/kg; lanes 5 and 7), DBcAMP (1 mg/kg; lanes 6 and 8), or MeS-ATP (5 mg/kg; lanes 9 and 10). DETC was given 30 min before autacoids or LPS. Lane 0, iNOS mRNA or iNOS protein standards; lane M, kilobase marker for iNOS mRNA. B: representative cERT-PCR and Western blot gels (50 µg of applied protein) showing effect of DETC (5 mg/kg it; lanes 2, 4, 6, and 8) on autacoid-induced enhancement of LPS-mediated iNOS mRNA and protein of AM from rats pretreated in vivo with DBcAMP (0.1 mg/kg it) or MeS-ATP (5 µg/kg it) 15 min before administration of LPS (0.6 mg/kg it). Lane 0, iNOS mRNA and cDNA or iNOS protein standards; lane 1, LPS in PBS-pretreated rats; lane 2, LPS in DETC-pretreated rats; lanes 3 and 4, DBcAMP in PBS- and DETC-pretreated rats; lane 5, DBcAMP facilitation of LPS in PBS-pretreated rats; lane 6, impairment in DBcAMP facilitation of LPS in DETC-pretreated rats; lane 7, MeS-ATP facilitation of LPS in PBS-pretreated rats; lane 8, impairment in MeS-ATP facilitation of LPS in DETC-pretreated rats; lane M, kilobase marker for iNOS mRNA and cDNA. For details see Fig. 1 legend.

Fig. 11. Left: effect of DETC on direct effects of LPS and autacoid mimetics on iNOS mRNA (top) and iNOS protein (bottom). DETC was given 30 min before PBS (0.5 ml/kg it), LPS (0.6 mg/kg it), DBcAMP (0.1 and 1 mg/kg it), or MeS-ATP (5 mg/kg it). Values are means ± SE from 6–14 experiments. Right: effect of intratracheal administration of DETC on PBS-, LPS-, DBcAMP-, and MeS-ATP-mediated changes in RNI levels in BAL fluid (top) and 1-h ex vivo incubates of rat AM (bottom). Values are means ± SE from 5–14 rats/group. *Significantly different (P < 0.05) from autacoid or LPS in absence of DETC. **Significantly different (P < 0.05) from response to LPS in presence of DETC.
iNOS protein can be detected. Induction of gene transcription is a prerequisite for the control of iNOS enzyme activity (17, 28), and iNOS mRNA is not expressed in the absence of the autacoids or LPS. Endogenous cAMP and ATP also exist in AM and should be capable of activating the machinery to prevent the degradation or stimulate upregulation of any constitutive iNOS mRNA. Despite the absence of iNOS mRNA and iNOS protein in AM from PBS-treated rats, iNOS mRNA and iNOS protein were increased in the AM 2 h after intratracheal administration of DBcAMP or MeS-ATP. The increase in iNOS mRNA in the AM from DBcAMP- and MeS-ATP-treated rats did not differ from that produced by LPS, which activates the transcription of iNOS mRNA and has only minor inhibitory effects on the degradation of iNOS mRNA (17, 28). Thus it is unlikely that the autacoids increase the levels of iNOS mRNA in rat AM in vivo by preventing its degradation.

Several P2y receptor agonists act synergistically with LPS to stimulate macrophage membrane guanosine 5’-triphosphatase (GTPase) activity (12). MeS-ATP is a partial agonist at the P2y receptor but is devoid of stimulatory effects on GTPase activity (12). Thus it is also unlikely that stimulation of membrane GTPase can explain the ability of these autacoid mimetics to upregulate the iNOS system and enhance the effects of LPS on the iNOS system of the rat AM in vivo. Finally, inhibition of protein synthesis with cycloheximide inhibits LPS- and cytokine-mediated upregulation of iNOS transcription (16, 23). Because cAMP, and possibly P2y receptor stimulation, can activate protein synthesis, it is possible that they upregulate a transcription factor or a protein promoter of transcription, which in turn activates the formation of iNOS mRNA (3, 17, 27, 28, 48) and, once formed, may subsequently prevent its degradation (39). Alternatively, each of the autacoid mimetics can upregulate protein kinase A (PKA), which may result in the activation of PKA-dependent transcription factors or inhibition of a repressor of transcription (3). This also may upregulate iNOS transcription (7). Further studies are required to test these postulates in vivo.

Differences between effects of autacoid mimetics and LPS on iNOS protein. Translation of iNOS protein was reduced in AM from the rats treated in vivo with DBcAMP and MeS-ATP compared with LPS-treated rats. Dimerization of the iNOS subunits is essential for the posttranslational activation of iNOS activity (9). Among the factors promoting dimerization are tetrahydrobiopterin (BH4), arginine, and heme (9, 22). LPS increases arginine transport and upregulates BH4 in AM (4, 17, 28). DBcAMP and MeS-ATP are devoid of these actions (17, 21, 28). Moreover, NO limits the dimerization of iNOS by diminishing the availability of heme iron to the enzyme (10, 17, 28). cAMP increases translation of iNOS mRNA to protein (17, 28). The low and high doses of DBcAMP were synergistic and additive, respectively, with LPS-mediated upregulation of iNOS protein, yet were additive with LPS-induced increases in the AM content of iNOS mRNA. Because LPS can upregulate BH4 and arginine transport to AM, we speculate that these factors may have contributed to the synergistic effect of DBcAMP and MeS-ATP on LPS-mediated upregulation of iNOS protein. Thus we speculate that the lower amounts of iNOS protein and subsequent generation of RNI measured in AM from rats treated with the autacoid mimetics may result, in part, from limited posttranslational dimerization of iNOS. The inability of MeS-ATP to synergize with LPS cannot be explained, unless the maximal rate of iNOS formation was reached at the endogenous concentrations of arginine in the AM (17, 28). Alternatively, it is possible that the autacoids and LPS induced two dis-
tinct isozymes of iNOS protein similar to those found in the rat kidney (35). The synergism between the low dose of DBcAMP- and LPS-mediated upregulation of iNOS protein in AM may have resulted from a sensitivity of the LPS-induced isozyme to DBcAMP but not to MeS-ATP. The mechanism of action of the autacoids on the iNOS system remains to be elucidated.

Similarities and differences between studies. Our findings that in vivo administration of DBcAMP and MeS-ATP upregulated transcription of iNOS mRNA and increased iNOS protein and RNI are in agreement with some in vitro studies using peripheral macrophages, fibroblasts, vascular smooth muscle cells, and cardiac myocytes in cell culture (6, 14, 24, 26, 29, 38, 39, 44). However, our results differ from those studies that only demonstrated inhibitory effects of these and related compounds when they are used in vitro in high concentrations with a combination of LPS and cytokines (8, 12, 17, 28, 37, 38). Moreover, our findings that DBcAMP- and MeS-ATP-induced upregulation of the iNOS system is independent of upregulation of NF-κB and refractory to inhibition by DETC differ from most data obtained in cell culture (6, 8, 12, 14, 17, 24, 26, 28, 29, 37–39, 44). Several factors can explain these differences, including the dose or concentration of the autacoid mimetics used and the cell type under study. However, the most important factor is probably the difference between the use of an in vivo and a cell culture model.

Dose and concentration effects. MeS-ATP is a partial agonist of P2y receptors, so low doses may act selectively as an agonist, whereas higher doses may act as an antagonist. Moreover, in vitro studies that demonstrated cAMP- and MeS-ATP-induced inhibition of LPS-mediated upregulation of iNOS mRNA and iNOS protein utilized high micromolar and millimolar concentrations of cAMP or DBcAMP or 0.1 mM MeS-ATP (8, 12, 37, 38). In contrast, low concentrations of prostaglandin E2 and DBcAMP increased iNOS mRNA in cultured RAW 264.7 macrophages and enhanced interferon-γ (34) and TNF-α-mediated upregulation of iNOS mRNA and iNOS protein (44). Thus low and physiological levels of cAMP and P2y receptor stimulation may upregulate the iNOS system, whereas high concentrations of these autacoids may inhibit the iNOS system.

Cell culture vs. in vivo studies and cell types. The use of cell lines and primary cells in culture may also modify the ex vivo cell-signaling pathways used to upregulate transcription of iNOS mRNA and its translation to iNOS protein. Peptide growth factors and the cell culture process itself cause many enzymes and proteins to revert to their fetal phenotype (5). Many cell lines and primary cells in culture exhibit phenotypic and genotypic transformations that may hinder the extrapolation of the cell-signaling pathways found to their tissues of origin in vivo (5, 43). For example, TNF-α is a potent inhibitor of endothelial cell growth in vitro but is angiogenic in vivo (42). Also, differences exist in the induction mechanisms for iNOS mRNA between rat aortic smooth muscle cells in culture and isolated aortic strips (43). Finally, LPS, DBcAMP, and MeS-ATP upregulate iNOS transcription and RNI production within 15 min after their administration in vivo (20, 21, 31, 45). Yet, continuous exposure for at least 10–48 h to a fixed concentration of these compounds is required to stimulate the iNOS system or to modify the effect of cytokines on the iNOS system (6, 8, 12, 14, 17, 24, 26, 28–30, 38, 39, 43). Thus the effects of these autacoids on the iNOS system may not be expressed in vitro, or their cell-signaling pathways utilized to induce iNOS mRNA and iNOS protein may differ when tested in vivo and in cell culture.

Conclusions. We conclude that at least two distinct cell-signaling pathways exist for in vivo induction of iNOS transcription in rat AM. The first is a cytokine- and LPS-stimulated pathway that involves stimulation of the transcription factor NF-κB, is inhibitable by DETC (15, 36), and is associated with activation of the inflammatory response. The second pathway can be...
activated by DBCAMP and the P2y receptor partial agonist MeS-ATP. It is not associated with upregulation of NF-κB, it is refractory to inhibition by DETC, and it does not elicit an inflammatory response in the lung. Speculatively, this second pathway may involve activation of PKA (21). DBCAMP and MeS-ATP administered with LPS enhance LPS-mediated upregulation of iNOS mRNA, iNOS protein, and NO in an additive and synergistic fashion. These data support the conclusion that in vivo induction of iNOS in rat lung AM may be selectively regulated by drugs and endogenous autacoids that increase cAMP or activate P2y receptors. Also, significant qualitative and quantitative differences appear to exist between rates and mechanisms of upregulation of the transcription of iNOS mRNA in vivo and in macrophages subjected to cell culture. This suggests that the process of cell culture may modify the cell-signaling pathway involved in the upregulation of iNOS, in agreement with the findings in isolated aortic strips and aortic smooth muscle cells in culture (43). The mechanism(s) by which cAMP and MeS-ATP increase iNOS mRNA and iNOS protein remains to be defined.

Perspectives

Although cellular regulation of rat and human iNOS mRNA and iNOS protein has many similarities, differences also exist (17, 28). Thus the findings reported here may be significant for veterinary and clinical medicine as well as for basic research. First, if cAMP and P2y receptor stimulation upregulate the transcription of iNOS in humans and higher mammals, then medications that upregulate these systems may also upregulate iNOS. This may be beneficial in asthma and hypertension, where drugs that elicit increases in cAMP or ATP will also stimulate low levels of iNOS, contributing to maintained dilation of airway or vascular smooth muscle without further stimulation of inflammation. However, it may also be deleterious in other heart and lung diseases in which oxygen free radicals are elevated, since this could result in high levels of the more toxic peroxynitrite (28, 32). Also, this finding makes it more difficult to determine the role of iNOS in diseased tissue in animals and humans. For example, NO in the exhaled air of asthmatic patients is derived primarily from constitutive NOS (49). iNOS is also increased in platelets from patients with coronary atherosclerosis (11) as well as transplanted hearts from patients with some forms of heart failure (17, 28). These patients usually are treated with medications that can modulate the cAMP and purinergic receptor pathways. Thus the existence of iNOS in these patients may not be related to the pathogenesis of disease but rather may result from the effects of medications on the iNOS system. Thus it is important that the effect of the drugs taken by the patients or tissue donors be considered in the design, execution, and evaluation of experiments when the iNOS system is evaluated. Finally, the cell-signaling pathways involved in iNOS regulation differ in aortic smooth muscle cells in culture and isolated aortic strips in a muscle chamber (43). Cell culture, in vitro, and in vivo models each have their strengths and weaknesses. However, when cell-signaling pathways are found in culture and/or in vitro, they may not represent the cell-signaling pathway that is active in vivo without rigorous testing and confirmation. This caveat applies not only to the iNOS system but to any system in which the cells undergo changes in phenotype or genotype in culture. Thus an understanding of the role of NF-κB and other transcription factors in vivo is needed to understand the physiological cell-signaling pathways involved in the regulation of iNOS in animals and humans and the sites of their dysregulation in disease states.

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