Lack of Inhibitory Effect of Dexamethasone on Exhalation of Nitric Oxide by Healthy Humans

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Nitric oxide (NO) is present in the breath exhaled from healthy humans. The location and subtype of the NO synthase responsible for the expired NO are unknown. As dexamethasone inhibits the induction of NO synthase, we evaluated the effect of administering dexamethasone (4 mg/day for 2 days) on the amount of NO exhaled by eight healthy men. The amount of NO showed a significant linear correlation with the duration of exhalation, allowing the rate of NO release to be calculated. The rate of NO release was 0.047±0.023 nmol/s before drug administration. There was no significant change in the release rate at the end of the 2-day administration of drug or at 5 days after cessation. Serum concentrations of interferon-γ and interleukin-1β were unaffected by the administration of dexamethasone. These results suggest that the NO released from the human airway under normal conditions is not generated by the action of inducible NO synthase.

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Introduction

Nitric oxide synthase (NOS) catalyzes the conversion of L-arginine to nitric oxide (NO). Three isoforms of NOS have been isolated, cloned, and sequenced. These isoenzymes can be divided into two subtypes based on activity (1). Constitutive NOS (cNOS) is expressed by endothelial and neural cells and is stimulated as a result of cell receptor activation. Inducible NOS (iNOS) is induced in macrophages and other cells in vivo or in vitro by cytokines or lipopolysaccharides. Glucocorticoids inhibit the induction of iNOS via a receptor-mediated event that involves the inhibition of the synthesis of mRNA for de novo synthesis of iNOS (1, 2).

Endogenous NO, generated by the action of NOS (3, 4), is present in the breath of normal humans at ~10 parts per billion (ppb) (3, 5, 6), and at even higher levels in asthmatic patients (4, 7). Immunohistochemical studies have demonstrated the presence of NOS in the lung, specifically in the pulmonary vascular endothelial cells (8, 9), nerves (9, 10), alveolar macrophages (9, 11) and airway epithelial cells (9, 12). Nevertheless, the regulation of NO metabolism in the lung and airways is not well understood.

Some types of cells in the respiratory system can demonstrate iNOS activity. While there is no conclusive proof that NO is formed from L-arginine in human macrophages (13), there is indirect evidence of NO formation in alveolar macrophages (11, 14). Alveolar macrophages from normal subjects also contain mRNAs for cytokines, specifically IL-1β (15). Human macrophages that have matured in vitro release substantial amounts of nitrite, the metabolite of NO, even in the absence of overt stimulation (16).

The epithelium mediates the interaction between the lung and inhaled oxidants. Oxidative stress induces iNOS in human epithelial cells via the induction of the transcription factor (17). The combination of IL-1β, IFN-γ, and TNF-α induces iNOS in murine lung epithelial cells (18) and in rat type II pneumocytes (19). In the latter instance, the production of NO is increased after the inhalation of a pulmonary irritant. Marked iNOS immunoreactivity has been detected in the epithelium of histologically normal human airways and occasionally, in the alveolar macrophages and endothelium (9). Hamid et al detected iNOS immunoreactivity in the epithelium and in some inflammatory cells in the lungs of individuals with asthma. They also observed immunoreactivity, although less frequently, in the nonasthmatic controls (12). Alving et al suggest that NO in exhaled air originates mainly in the nasopharyngeal airways (7). This hypothesis has been supported by Gerlach et al, who suggest that bacteria or bacterial toxins may stimulate the nasal
mucosa to synthesize NO (20).

It is not known which NOS subtype is responsible for the major portion of NO exhaled by healthy humans, but the above observations suggest that iNOS may be active in normal human alveolar macrophages, lung epithelium, or nasal airways under basal conditions. Under conditions in which iNOS is expressed, most of the NO produced is due to iNOS activity (1, 2). Levels of NO in exhaled air vary more than 10 fold among normal individuals without respiratory symptoms (unpublished observations). This wide variation indicates that iNOS may contribute to the production of NO in the exhaled air of apparently healthy people. In the present study, dexamethasone was administered to healthy subjects to block the induction of iNOS (21). The effect of such dexamethasone administration on the amount of exhaled NO was measured.

Materials and Methods

Subjects

Eight Japanese male volunteers aged 33±8 years were enrolled in the study. They had no symptoms of respiratory disease and none was receiving medication. Subjects who smoked (n=4) were asked to refrain from smoking for at least 1 hour before the initiation of the study. The study was approved by the institutional committee on human research. Informed consent for participation was obtained from each subject.

Protocol

Each subject was allowed to sit and breathe normally for 10 minutes, after which the blood pressure was measured with a sphygmomanometer. Samples of venous blood and exhaled air were collected as described below. Dexamethasone (Corson; Takeda Yakuhin, Osaka) was administered at a dose of 1 mg every 6 hours for 2 days. Within 4 hours after, and again at 5 days after the last dose of dexamethasone, blood pressure was re-measured and specimens of venous blood and exhaled air were collected.

Collection of exhaled air samples

Room temperature was maintained at ~25°C and the relative humidity at <60%. After inspiration through the mouth, air was exhaled through the nose. Nose clips were not used to allow air in the nasopharyngeal space to easily diffuse into the atmosphere during the exhalation. Under specific instructions, the subject exhaled through a mouthpiece connected to a 6 liter bag made of polyvinylfluoride film (Tedlar bag; Iuchi, Tokyo). A Teflon column (15 x 50 mm) packed with 6.7 g of anhydrous silica gel (particle size, 1.7 to 4.0 mm; Kanto Chemical, Tokyo) was inserted between the mouthpiece and the Tedlar bag. The silica gel dried the exhaled air sufficiently to prevent the condensation of vapor on the walls of the Tedlar bag, even after the exhalation of 10 deep breaths. Nitric oxide was not adsorbed by the silica gel and was stable in the Tedlar bag for several hours if the sample remained dry and Tedlar bags containing samples were protected from light until analysis. In four calibration trials, air containing NO at 10 ppb was passed through the silica gel column. The concentrations measured after 0, 2, 4, and 6 hours in the bag were 9.9±0.1, 9.9±0.1, 9.8±0.2, and 9.5±0.3 ppb, respectively. When humid air was exhaled directly into the bag, the concentration of NO decreased by ~20% within 30 minutes (from 11.2±3.4 to 8.8±5.2 ppb, n=4). The study was performed only when the concentration of NO in room air was <3 ppb.

NO gas analysis and rate of NO release in airway

Nitric oxide concentration was measured with a chemiluminescence analyzer (Model GLN-32; Denki-Kagaku-Keiki, Tokyo), that detects photons generated by the oxidation of NO by ozone. The photomultiplier signal was amplified and recorded with a recorder (Model 056; Hitachi, Tokyo). The flow rate for sampling was 800 ml/min, and the recorded curve for the applied sample reached steady state in 3 minutes. The minimum limit and the resolution for the detection of NO was 0.2 ppb. The coefficient of variation was 2% at 20 ppb NO. Before use, the instrument was calibrated by NO at 100 ppb in nitrogen (Sumitomoseika, Chiba) before use. Each gas analysis was performed within 1 hour after collection.

The relationship between the amount of NO in the exhaled air and the duration of exhalation was analyzed. Subjects were instructed to exhale air, after a full inspiratory effort, at six different speeds of exhalation that were determined arbitrarily by each subject from very fast to very slow. The volume of air was recorded in each instance and corresponded to the vital capacity. Subjects practiced the procedure several times. The order of the speed of exhalation was randomized. The six samples were collected in separate bags. More than 2 minutes elapsed between each sampling. The time from the start to the end of each sampling was measured with a stopwatch and ranged from 11.7 to 70.0 seconds, depending on the ability of the subject. The protocol was repeated 10 times within 3 weeks in one volunteer who had no symptoms of respiratory disease during the weeks. The amount of NO (nmol) in the exhaled air sample was calculated from b x V x 24.5^-1; where b (ppb) is the NO concentration of the exhaled air sample, V (liter) is the sample volume, 24.5 (liter) corresponds to the volume of 1 mol of ideal gas at 25°C at 760 mmHg. Because the amount of exhaled NO exhibited a positive correlation with the duration of exhalation, a simple linear regression model based on the least-squares method was applied. The slope was regarded as the rate of NO release from the airway.

Measurement of cytokines and cortisol

The serum concentration of IFN-γ was determined by a radioimmunoassay, as described previously (22). The serum concentration of IL-1β was determined with a radioimmunoassay kit (Human IL-1β IRMA; Medgenix, Fleurus, Belgium), as was serum cortisol (Gamma Coat Cortisol; Baxter, Stillwater, MN).

Statistical analysis

Data are expressed as means±SD. Statistical analysis utilized the repeated-measures analysis of variance. When the F
value was significant, Scheffe’s test was applied. A p value of <0.05 was considered statistically significant.

**Results**

The serum cortisol concentration was 12.4±3.6 mg/dl before the administration of dexamethasone, below the detection limit (1.0 mg/dl) at the end of the 2-day administration, and 11.3±2.2 mg/dl on the 5th day after administration. The individual blood pressure, pulse rate, and serum concentrations of sodium, potassium and chloride were all within the normal range, and did not vary significantly among the three points of measurement of the protocol. The concentration of NO in room air averaged 0.8±0.6 ppb (0–2.5 ppb) immediately before initiation of the protocol. On rainy days, the concentration of NO in room air often exceeded 3 ppb. The protocol was not performed until the NO concentration in the room dropped to less than 3 ppb. The NO concentration of the air samples exhaled fastest all exceeded the NO concentration in room air.

There was a significant positive correlation between the duration of exhalation and the amount of exhaled NO (Fig. 1). The slope (nmol/s) of the linear regression relation between the amount of NO exhaled and the duration of exhalation was taken as the rate of NO release in the airway. This rate was not significantly affected by the administration of dexamethasone for 2 days (0.047±0.023 nmol/s before administration, 0.045±0.017 nmol/s at the end of administration, and 0.043±0.015 nmol/s 5 days afterwards) (Fig. 2). There was no significant difference in the rate of NO release between the smokers and nonsmokers. The rate of NO release was constant (0.035±0.004 nmol/s) in the one subject who repeated the protocol 10 times over a 3-week period.

The administration of dexamethasone did not affect the serum concentration of IFN-γ (0.9±0.2 U/ml before administration, 0.8±0.2 U/ml at the end of administration, and 0.9±0.2 U/ml 5 days after the cessation of administration). There was no correlation between the serum concentration of IFN-γ and the rate of NO release (p=0.69, 0.31, and 0.29, respectively). The serum concentration of IL-1β was below the limit of detection in our analysis (10 pg/ml) at all time points.

**Discussion**

In healthy humans, a two-day regimen of oral dexamethasone did not affect NO release by the respiratory system. Massaro et al (23) reported that parenteral administration of glucocorticoid (180–240 mg methylprednisolone over 4–24 hours, or a single 40 mg dose of prednisolone) in patients with acute asthma causes a reduction in expired NO. This decrease was evident as early as 48 hours after the initiation of therapy (180–240 mg of methylprednisolone over 4–24 hours). The antiinflammatory potency of dexamethasone is 5-fold higher than that of methylprednisolone and 6.25-fold higher than that of prednisolone (24). The biological half-life of dexamethasone (36 to 72 hours) is larger than that of methylprednisolone or prednisolone (12 to 36 hours) (24). Although the dose of dexamethasone administration in the present study had no more than half the antiinflammatory potency of the steroid treatments reported by Massaro et al, the extended period of administration and the prolonged biological half-life of dexamethasone should have compensated for the reduced dosage with respect to the inhibition of iNOS activity.

Cytokines increase the expression of iNOS mRNA in a dose- and time-related manner. This effect is mediated by transcriptional activation and is followed by the de novo appearance of iNOS protein (25). The half-life of iNOS mRNA is ~6 hours in cultured cells (26). Studies in vivo have shown that the induction of iNOS causes mRNA levels to peak at ~6 hours and elevated levels of mRNA persist for 24 hours in the rat mesentry (27) or 18 hours in the rat myocardium (28). It has also been shown that, after iNOS has been induced, the release of NO may continue for several days (2, 21, 29). In the present study, the rate of NO release in the one volunteer tested periodically remained constant over a period of weeks. These observations support the hypothesis that iNOS activity is relatively constant under basal conditions as a result of a balance between degradation and cytokine-induced synthesis. Dexamethasone treatment over a period of 2 days would be expected to reduce iNOS activity and, consequently, reduce NO production in the total respiratory system, only if iNOS activity made a significant contribution to the expired NO.

The site of origin of NO in the respiratory system remains controversial. Persson et al have suggested an airway origin, specifically the terminal and respiratory bronchioles, based on their observations of a NO peak in single exhalations (6, 30). It has also been suggested that NO in exhaled air originates mainly in the nasal airways (7, 20). However, if NO was diffusing from the nasopharynx, the concentration of NO would be highest in the dead space. It has been shown that measurement of NO and carbon dioxide exhibit the same delay before increasing after the beginning of a single oral exhalation (6). Air is prevented from leaking from the nasopharynx into the oral cavity by the soft palate. The soft palate rises, and moves backward against the pharynx, to thus close off access to the nasal space above (31). It is reasonable to assume that pulmonary exhaled gas was relatively uncontaminated by nasopharyngeal gas.

The fact that the administration of dexamethasone did not affect NO release from the respiratory airways indicates that the NO exhaled in normal subjects is unlikely to be generated as a result of the activity of iNOS localized in the airway. This result supports the observation of Kharitonov et al (4) that in asthmatic patients, inhaled steroids can reduce the amount of NO to values seen in normal individuals (but not to lower values). There appears to be a basal level of exhaled NO which is not influenced by the administration of steroids. It is of interest to note that the subject with the highest NO values before administration of dexamethasone (no. 5 in Fig. 1) had a significant (~40%) drop in exhaled NO after treatment. This profile suggests that the synthesis of iNOS had been induced in this patient and was inhibited by dexamethasone.

A simple model yielding the concentration (C, mol/liter) of...
Dexamethasone and NO Exhalation

Figure 1. The relation between the amounts of NO exhaled and the duration of exhalation in each subject before (○), at the end of (□), and 5 days after (△) administration of 8 mg of dexamethasone over 2 days. Single regression analysis with the least-squares method revealed a significant correlation between variables (p<0.013 for all subjects at all points).

The rate of NO release refers to the rate of elimination or expiration, but not the rate of NO production, in the airway. The NO release rate and NO production rate would be equal only if it were known that no NO was scavenged by the lung tissue or by capillary red blood cells. Uptake of exogenous NO from alveolar air, probably by capillary red blood cells, has been demonstrated (32). Breath-holding causes an elevation of NO gas in the sampling container after time (t, sec) was derived as follows: C=P x t x 1/V, where P (mol/sec) was the average NO release over time and V (liter) was the air volume collected. When the equation was converted to C x V=P x t, where C x V corresponds to the amount of NO (mol) in the exhaled air sample and P corresponds to the slope versus time, the rate of NO release (P) was found to be constant because the amount of NO in the sample (C x V) showed a linear relation to the duration of exhalation (Fig. 1).
release in the airway could be estimated.

The vascular endothelium also expresses iNOS in response to exposure to cytokines such as IFN-\(\gamma\)(33). The iNOS activity of vessels may thus be associated with circulating concentrations of cytokines. Borland et al have suggested that pulmonary endothelium-derived NO may enter the airspace in sufficient amounts to allow its measurement in exhaled air (34). The lack of correlation between the serum concentration of IFN-\(\gamma\) and the NO release rate in the present study suggests that pulmonary endothelium-derived NO generated by the action of IFN-\(\gamma\) does not contribute significantly to released NO.

The results of this study suggest that, in healthy individuals, NO generated by iNOS makes a negligible contribution to the total exhaled NO. The NO present in expired air is probably generated by cNOS in non-alveolar regions of the respiratory system.

References

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