Free Radicals (Nitric Oxide)

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Identification and characterization of the functions of free-radical nitric oxide (NO) as an endogenous mediator of biological effects represent a relatively recent development in biomedical science. The description of enzymatically controlled conversion of L-arginine to citrulline and NO by nitric oxide synthase was presented in 1987. The groundwork for intense research toward the identification of the enzyme was laid by pharmacological studies on the role of endothelium in the control of vascular tone and by recognition of nitrate synthesis in both humans and animals, which increases upon immunostimulation. Endogenously formed NO has been shown to be the common mediator of both endothelium-dependent relaxation and cytotoxic effects of activated macrophages and neutrophils.

The recognition of NO as an effector molecule and the identification of the endogenous pathway of its production provided researchers with powerful tools to probe the functions, reactivity, and effects of NO in a wide array of cellular systems under physiological or pathological conditions. Indeed, the period between 1987 and now registered unprecedented growth in the research on the biological functions of NO, and it is now well established that such functions encompass a much greater spectrum of effects spanning beyond the regulation of vascular tone or mediation of cellular immunity (reviewed in refs F1–F4).

This review will focus on the developments pertinent to the laboratory monitoring of NO-mediated processes. The emphasis will be placed on approaches that have the potential of being utilized in clinical laboratory medicine and on studies that advanced our knowledge in laboratory evaluation of disease processes. Methodological advancements will be noted as appropriate. A brief account of recently developed therapeutic applications based on targeted manipulation of NO metabolism will also be presented.

The literature search was concluded in October of 1994.

ENDOGENOUS SYNTHESIS OF NO

Several different isoforms of nitric oxide synthase (NOS) have been identified in different cells and tissues (*F5*, *F6*). They can be broadly categorized as constitutive and inducible forms. Tissues containing the constitutive types of NOS include vascular endothelium, platelets, and certain neurons of both the central and peripheral nervous system. The constitutive enzyme produces relatively low quantities of NO, and its activity is regulated by intracellular levels of calcium ions. The primary biological roles of NO generated by constitutive NOS are the regulation of vascular tone, inhibition of platelet aggregation, and synaptic transmission. These effects are exerted by NO-mediated stimulation of guanylate cyclase in the target cells with concomitant rise in intracellular levels of cyclic guanosine monophosphate (cyclic GMP).

NO produced by inducible NOS is involved primarily in the mediation of the cellular immune response. The expression of the enzyme requires the exposure of cells to endotoxins and/or certain cytokines. The production of NO is detected after a lag phase of approximately 4–6 h after the exposure, reflecting de novo synthesis of enzyme molecules. The induction of the enzyme is inhibited by glucocorticoids (*F7*). Inducible NOS generates higher amounts of NO relative to the constitutive form, and the synthesis is independent of intracellular calcium levels. NO-mediated effects on target cells include coordination to metalloproteins and inhibition of DNA synthesis with resulting growth arrest and cellular destruction. Besides activated macrophages and neutrophils, the expression of inducible NOS can be experimentally documented in a growing list of other cell types, including smooth muscle cells, hepatocytes, astrocytes, pancreatic β cells and several types of tumor cells.

Several derivatives of L-arginine with substitutions on the nitrogen atom of the guanidino group, such as N^G-monomethyl-L-arginine (L-NMMA), N^G-nitro-L-arginine methyl ester (L-NAME), and N^G,N^G dimethyl-L-arginine (asymmetric dimethylarginine, ADMA) act as inhibitors of NOS. These inhibitors do not discriminate between inducible and constitutive forms of NOS. The only compound with selective inhibitory effect is aminoguanidine, being approximately 10–100 fold more potent in inhibiting the inducible NOS (*F8*). The inhibitory effects of ADMA are not shared by its symmetric counterpart $N^G,N^{G'}$-dimethyl-L-arginine (symmetric dimethylarginine, SDMA) (*F9*, *F10*).

Some of the guanidino-substituted derivatives of L-arginine, in particular ADMA, L-NMMA, and SDMA are produced endogenously and are readily identifiable in biological fluids (*F11*). The manipulation of dietary intake of L-arginine or protein was found to have little effect on the urinary levels of these compounds, supporting the contention that substituted arginines in biological fluids are derived primarily from the catabolism of endogenous proteins (*F11*). The presence of methylated arginines was subsequently demonstrated in myelin basic protein and certain nuclear proteins (*F12*, *F13*). The presence of methylated arginines in proteins results from posttranslational enzyme-catalyzed methylation of L-arginine utilizing *S*-adenosylmethionine as a donor of a methyl group (*F14*, *F15*).

After release from proteins, a portion of the substituted arginines can be enzymatically hydrolyzed to L-arginine. One of the enzymes catalyzing such a hydrolysis, N^GN^G-dimethylarginine dimethylaminohydrolase (DDAH), with hydrolytic activity toward ADMA and NMMA, but not SDMA, has been purified from rat kidney (*F16*). Antibodies raised against the purified enzyme were subsequently used for the evaluation of distribution of DDAH in different tissues. In rat, high levels of DDAH have been found in kidney, pancreas, brain, liver, and aorta (*F17*). These findings suggest that the enzymatic hydrolysis of methylated derivatives of L-arginine may participate in the regulation of NOS activity by controlling the endogenous levels of NOS inhibitors.

Plasma levels of ADMA were shown to rise in renal failure proportionally to the increase in creatinine. The accumulation of ADMA in plasma was proposed to account for some of the pathological features of this disorder (*F18*). This contention was supported by findings that plasma levels of ADMA in patients with renal failure were comparable to the concentrations of ADMA required for inhibitory effects on NO synthesis in both in vitro and in vivo models. Plasma levels of ADMA in control subjects were insufficient for eliciting the inhibitory effects (*F9*, *F18*).

ANALYSIS OF NO AND ITS METABOLITES

Authentic NO. The detection of authentic NO in biological systems can be done either by NO-sensitive probe or by chemiluminescence-based assay. A NO-sensitive probe consisting of a porphyrinic polymer deposited on a tip of carbon fiber permits in situ detection of NO production. The sensitivity of the probe is sufficient to detect agonist-induced generation of NO in single cell (*F19*).

The chemiluminescence assay, originally developed for the analysis of NO in gaseous samples, is based on generation of a chemiluminescent intermediate by reaction between NO and ozone. Application of this procedure to liquid samples requires purging of NO from the sample, typically by a stream of inert gas. Alternative sampling can be done by analyzing headspace gas (*F20*). If the analysis is carried in the presence of a reducing agent in acidified samples, in addition to authentic NO, the signal will include NO released from its labile adducts (nitrosoamines, nitrosothiols) and nitrite. The chemiluminescence signal is relatively specific for NO (*F21*); however high levels of ammonia may introduce positive bias (*F22*).

An alternate chemiluminescence assay utilizes conversion of NO to peroxynitrite. The chemiluminescence signal is generated by the reaction of peroxynitrite with luminol (*F23, F24*).

The detection of NO by the above techniques is applied primarily in pharmacological studies of NO metabolism using cell cultures, organ/tissue perfusion, or subcellular components as experimental models. In clinical studies, chemiluminescence-based detection of NO has been used in the analysis of exhaled air. In lungs, NO is generated by several cell types, including alveolar macrophages and epithelial cells. It was observed that the amount of NO in exhaled air was approximately 3-fold higher in patients with asthma compared to healthy controls (*F25*). Such an elevation was absent in asthmatic patients receiving inhaled glucocorticoids. The amount of NO in exhaled air was reported to be decreased in smokers (*F26*), apparently reflecting down-regulation of endogenous alveolar NO production due to inhalation of low doses of NO present in tobacco smoke.

The detection of authentic NO in blood is of limited value due to rapid reactivity of NO with blood constituents (see below). Using a porphyrinic microsensor, it was shown that the signal produced by adding NO (40 μM) to stirred blood almost completely dissipated within 5 s (*F27*).

Adduct of NO with Metalloproteins. Metalloproteins, including proteins containing iron–sulfur centers, represent an important biological target of NO. Formation of coordination bonds between NO and metal centers results in altered functional properties of proteins. Several well-recognized interactions of this type include inhibition of aconitase and enzymes of mitochondrial respiratory chain. Reactions with metal centers of metalloproteins account for most part for the cytotoxic effects of NO (*F28, F29*).

Many of the complexes of NO with metalloproteins are paramagnetic and are amenable to detection by electron spin resonance spectroscopy (reviewed in refs F30 and F31). The formation of paramagnetic iron–nitrosyl complexes has been identified both in vitro and in vivo under conditions characterized by upregulation of inducible NOS, such as cell-mediated immune response to syngeneic tumors (*F32*), interleukin-1-induced pancreatic dysfunction (*F33*), or allograft rejection (*F34*). In addition to the involvement of iron–nitrosyl complexes in the cytotoxic effects of NO mediated by high-output inducible NOS, there is evidence to suggest that such complexes may be formed in endothelial cells and may serve as transient intermediates capable of NO release (*F35*).

Paramagnetic complexes formed by the reaction of NO with hemoglobin have received considerable attention recently for their potential utility for in vivo monitoring of NO production. Hemoglobin, being the most potent scavenger of NO in blood, reacts with NO in two principal modes related to oxygen saturation (*F36–F39*): In oxygenated arterial blood, the reaction of oxyhemoglobin with NO results primarily in the formation of methemoglobin and nitrate. Under conditions of low oxygen saturation (such as in venous blood), NO reacts with deoxyhemoglobin by coordinating to ferrous atoms of heme. The resulting adduct, nitrosylhemoglobin (HbNO), is paramagnetic and exhibits characteristic spectral signal when analyzed at 77 K (*F21, F40*).

The attempts to monitor the circulatory levels of NO by detection of HbNO complexes remain at the present time only moderately successful. In the absence of a stimulus for elevated NO production, the circulatory levels of HbNO are usually below the detection limit of the assay. The appearance of HbNO in the circulation occurs after upregulation of inducible NOS, such as in the animal models of septic shock (*F41*). In a sample of patients receiving nitroglycerin [which in a manner similar to other nitrovasodilators is metabolized to NO (*F42*)], HbNO was identified in only about 50% of subjects (*F43*).

The inherent limitation of using hemoglobin as an endogenous spin trapping agent for detection of NO production relates to the above outlined differences in the reactivity of NO with oxyhemoglobin and deoxyhemoglobin. The relative proportions of HbNO to methemoglobin formed under in vivo conditions will be influenced by the oxygen saturation at the site(s) of NO interaction with hemoglobin. The level of oxygen saturation at those sites is difficult to predict in in vivo models; furthermore, the saturation may change with experimental manipulations. For example, the comparison of plasma levels of nitrate to blood levels of HbNO in the animal model of septic shock showed that the increases in plasma nitrate were approximately 1 order of magnitude higher compared to increases in HbNO (*F44*). Furthermore, HbNO is present in the circulation in several conformational states, resulting in the formation of heterogenous spectral signals (*F45*).

An alternate way of detecting NO production in vivo is the administration of exogenous spin trapping agent. Subcutaneous injection of divalent iron complexed with *N*-methyl-D-glucamine dithiocarbamate to mice with experimentally induced septic shock permitted the evaluation of real-time generation of NO by monitoring the appearance of paramagnetic complexes in blood of conscious animals (*F46*).

Nitrate/Nitrite. In aerobic aqueous solutions at physiological pH, NO is converted primarily to nitrite (*F37*). However, on a quantitative basis, the extend of conversion of NO to nitrite in blood is only marginal in comparison to its oxyhemoglobin-mediated conversion to nitrate. As a result, serum levels of nitrate in healthy subjects are approximately 2 orders of magnitude higher relatively to nitrite (*F40, F47*).

The potential utility of the measurement of serum (or urinary) nitrate in evaluating NO metabolism in physiological or pathological states has been examined in several recent studies. This development has been accompanied by refinement of methodologies for detection of nitrate in human biological fluids. Besides the traditional techniques using either cadmium reduction of

nitrate to nitrite followed by determination of nitrite by diazotization reaction (*F47*) or liquid chromatography (*F48*), several newer techniques based on capillary electrophoresis (*F49, F50*), fluorometry (*F51*), or enzymatic reduction by nitrate reductase (*F52, F53*) have recently become available.

Because of the low concentration of nitrite relative to nitrate in biological fluids, some of the methodologies applied to the analysis of serum or urine were based on quantitative conversion of nitrate to nitrite followed by the determination of total nitrite concentration. Results obtained by such assays will be hereafter referred to as nitrate/nitrite.

Serum levels of nitrate/nitrite were found to rise during the follicular phase or after estrogen therapy (*F54*). The serum concentrations of nitrate/nitrite in the late follicular phase in healthy females were approximately 70% higher compared to concentrations in the early follicular phase and were significantly correlated with increases in serum concentration of 17-β-estradiol. No correlation was found between serum levels of nitrate/nitrite and progesterone. It was proposed that the estrogen-induced upregulation of NO synthesis may be involved in the cardioprotective effects of estrogens in females during reproductive age. Similarly, the elevation of plasma and urinary nitrate/nitrite was observed during pregnancy in an animal experiment model (*F55*), implicating the alterations in NO metabolism as one of the adaptative hemodynamic/immune changes associated with pregnancy. Whether insufficient upregulation of NO-mediated processes during pregnancy may result in preeclampsia/eclampsia remains to be elucidated. In support of such a contention are findings that the inhibition of NO synthesis in pregnant rats results in hypertension and retardation of fetal growth (*F56*).

Plasma or urinary levels of nitrate are characteristically elevated during immunostimulation as a result of NO generation by inducible NOS (*F57*). The predictive value of plasma nitrate/nitrite for progression into septic shock was evaluated in a sample of 20 newborn patients with sepsis (*F58*). None of the 12 patients with plasma nitrate/nitrite concentrations of <200 μmol/L developed septic shock whereas out of 8 patients with plasma nitrate/nitrite of >200 μmol/L, 6 progressed into septic shock.

Similarly, immunostimulation after allograft transplantation is associated with enhanced output of NO. In the animal model of orthotopic small bowel transplantation, serum nitrate/nitrite in allogeneically grafted animals was elevated approximately 8-fold at the sixth postoperative day, while no increases were seen in animals receiving a syngeneic graft (*F59*). Administration of cyclosporin to animals with allografts resulted in suppression of serum nitrate/nitrite levels; however, recurrence of rejection after discontinuation of cyclosporin therapy was associated with elevated nitrate/nitrite. These findings indicate that the laboratory evaluation of NO metabolism may provide a useful adduct in postoperative monitoring of transplant-receiving patients (*F60*).

The semiquantitative detection of urinary nitrite by dipstick is traditionally used as a diagnostic test of urinary tract infection. The presence of nitrite in urine has been regarded to result from bacterial production. The recognition of NO as a mediator of the immune response may require rectification of this assumption. The source of nitrite in infected urine seems to be in major part NO generated by infiltrating leukocytes (*F61*).

The evaluation of NO metabolism based on nitrate/nitrite measurement is not without limitations. Besides NO produced endogenously by NOS, the concentration detected in plasma or urine comprises nitrate/nitrite from diet, bacterial production, and inhaled NO. The contribution of dietary sources of nitrate was illustrated by findings that a combination of fasting with intake of deionized water resulted in approximately 50% decline in plasma nitrate level (*F49*).

Judged by previously reported values for healthy subjects, the interindividual variation in nitrate/nitrite represents approximately 30% (expressed as CV) for both plasma and timed urine specimens, indicating that the detection of subtle changes in NO production may escape detection in small-scale population-based studies. For example, manipulation of blood pressure or inhibition of NO synthesis in adrenalectomized rats resulted in changes in urinary excretion of nitrate/nitrite not exceeding 25–30% of control levels (*F62*). Long-term administration of NOS inhibitor to rats in a dose sufficient to induce hypertension and cardiac hypertrophy was associated with only 5–20% reduction in urinary nitrate excretion (*F63*).

Another source of complications may arise from population differences in the basal levels of nitrate. Small-scale studies conducted on healthy subjects in the United States and Europe reported mean basal levels of nitrate in plasma or serum of approximately 30–40 μmol/L (*F47, F49, F52, F57*). Large-scale study enrolling 205 healthy office workers in Japan (*F48*) found significant differences in the serum nitrate levels between males (97.0 ± 9.38 μmol/L; mean $\pm$ standard error; $n = 126$) and females (58.8 ± 5.41 μmol/L; $n = 79$); the mean value in males being more than 2-fold higher as compared to estimates based on the U.S. population. Using multiple regression, serum levels of nitrate in this study were found to be positively correlated with age and systolic blood pressure in males, while in females positive correlation was found with age and serum levels of triglycerides and cholesterol.

The above considerations indicate that the primary utility of nitrate measurements in biological fluids may be in clinical situations characterized by excessive production of NO (such as immunostimulation), resulting in robust increases in nitrate/nitrite. On the other hand, measurements of nitrate in situations characterized by deficient production of NO or by small changes in the activity of constitutive NOS are unlikely to be contributory unless other variables are carefully controlled.

S-Nitrosothiols. NO reacts readily with thiol groups of amino acids, peptides, or proteins forming nitrosothiol adducts. These compounds are capable of simulating many of the biological effects of NO, apparently by metabolically controlled generation of NO (*F64–F67*). S-Nitrosylation of homocysteine has been proposed to counteract its atherogenic and thrombogenic effects (*F68, F69*).

At the present time, there is only sparse information about the presence and function of *S*-nitrosothiols in the circulation. Using citrate plasma from five healthy volunteers, the total level of nitrosothiols has been found to be 7.19 ± 5.73 μmol/L (mean $\pm$ standard deviation) with more than 90% of the total content being attributed to *S*-nitrosoproteins (*F70*). The assay of total *S*-nitrosothiols can principally utilize metal ion-catalyzed or photolytic decomposition of *S*-nitrosothiols coupled either with detection of liberated NO or with spectrophotometric monitoring of decrease in absorbance at 336 nm (*F70–F72*). The analysis of individual nitrosothiols can be carried out by chromatographic techniques (*F70*) or by capillary electrophoresis (*F73*).

Cyclic GMP. As was indicated before, the biological effects of NO in the regulation of vascular tone, platelet aggregation, and

neurotransmission are mediated by increased levels of cyclic GMP in target cells. Although NO-mediated elevation of cyclic GMP has been reported in numerous experimental models, its utility as an indirect measure of in vivo NO synthesis is only sparsely documented. In pregnant rats, elevations in plasma and urinary nitrate/nitrite were accompanied by a similar rise in cyclic GMP levels (*F55*). Cardioprotective effects of NO donor FK 409 in an animal model of myocardial infarctions were reported to be correlated with increases in plasma cyclic GMP (*F74*).

One of the difficulties in interpreting the plasma or urinary cyclic GMP levels relates to the low specificity of this analyte, as the circulatory levels are influenced by several other endogenous mediators, atrial natriuretic peptide being the most prominent.

NO AND OXYGEN-DERIVED FREE RADICALS

It is well documented that the biological effects of NO are potentiated in the presence of superoxide dismutase, implicating superoxide as a potent scavenger of NO (*F75, F76*). Conversely, reaction of superoxide with NO would be expected to decrease the intermediate levels of superoxide and limit its availability for entering the cascade of reactions leading to lipid peroxidation and other untoward effects. Several lines of evidence have been recently presented in support of such a contention. NO was found to decrease the production of superoxide in activated human leukocytes (*F77*). Upregulation of NO synthesis in murine macrophages was accompanied by decreased potential for oxidative modification of low-density lipoproteins (LDL) (*F78–F80*).

NO-mediated inhibition of lipid peroxidation due to interaction with superoxide assumes that the reaction product of NO and superoxide is a relatively stable product which does not yield itself to further transformation into free-radical species. Under certain conditions, this assumption may not be valid. The intermediate product of reaction between NO and superoxide is peroxynitrite ($O_2^- + NO \rightarrow ONOO^-$), a relatively stable product when present in ionized form ($pK = 6.8$). Upon protonation, peroxynitrous acid can either isomerize to nitrate or form free-radical species with reactivities similar to hydroxyl radical (*F81*). Studies using synthetic peroxynitrite under in vitro conditions showed that peroxynitrite acts as a strong oxidant of sulfhydryl groups (*F82*) and initiates lipid peroxidation (*F83, F84*).

Several cell types, including endothelial cells and activated macrophages, are known to generate both NO and superoxide, and the formation of peroxynitrite by these cell types has been observed under in vitro conditions (*F85, F86*). Some pathological states characterized by disruption of the natural balance between prooxidants and antioxidants may be associated with enhanced production of peroxynitrite and the ensuing deleterious effects of this intermediate. In the animal model of ischemia/reperfusion injury, the toxic effects of reperfusion were suppressed by concomitant administration of NOS inhibitor and antioxidants, implicating peroxynitrite as an intermediate in prooxidant effects of NO (*F87*).

One of the distinct reactivities of peroxynitrite is the nitration of phenolic compounds (*F88*). The presence of 3-nitrotyrosine was detected in serum and synovial fluid from inflamed joints in patients suffering from active rheumatoid arthritis, while this analyte was absent in sera of control subjects (*F89*). These findings are consistent with the notion of increased formation of peroxynitrite during active inflammation.

The crucial question in our current understanding of the interaction of NO with oxygen-derived free radicals relates to the reconciliation of above discussed findings of both antioxidant and prooxidant effects of such interactions. It seems likely that the elucidation of mechanisms governing the reactivities of NO with superoxide (and/or other active molecules) within the biological microenvironment will require consideration of a multitude of variables, including the individual rates of generation and degradation of both radicals, redox status, simultaneous occurrence of additional competing reactions, and functional integrity of antioxidant defense mechanisms (*F90–F92*).

NO-MEDIATED PROCESSES AND ATHEROSCLEROSIS

Elevated LDL cholesterol is a well-recognized risk factor for the development of atherosclerosis. Studies on the vasorelaxing effects of endothelium-derived NO have provided significant insight into potential mechanisms of cholesterol-induced endothelial dysfunction. Some of the observations in this area will be briefly outlined below.

In vitro contractile studies on isolated arteries showed that the endothelial regeneration following physical injury was accompanied by decreased responsiveness of the endothelium to certain agonists capable of stimulating NO production and eliciting an endothelium-mediated relaxing response. Such an endothelial dysfunction was found to be due to an altered signaling pathway at the level of membrane-bound GTP-binding protein (*F93*) which couples the membrane receptor to intracellular processes. Similar alterations in GTP-binding protein were found in blood vessels of experimental animals fed a high-cholesterol diet (reviewed in F94). Inquiries into the mechanisms whereby elevated cholesterol modifies the GTP-binding protein provided evidence that the alteration in the signaling pathway can be simulated by the exposure of endothelium to oxidatively modified LDL (*F94*). Oxidative modification of LDL elicits several changes in both the lipid and apoprotein moiety of LDL particles, including conversion of lecithin to lysolecithin. Exposure of endothelium to lysolecithin mimicked the impairment of endothelium-dependent relaxation elicited by oxidized LDL (*F95, F96*).

In addition to aberrant receptor coupling, the downregulation of endothelial production of NO in hypercholesterolemia may be related to altered metabolism of endogenously produced inhibitors of NOS. Significant elevation of plasma levels of ADMA (9-fold comparing to controls) was observed in experimental animals fed a high-cholesterol diet (*F97*). Such an elevation could not be attributed to altered renal function as judged by plasma creatinine. Hypercholesterolemia in these animals was shown to be associated with the formation of atherosclerotic plaques. Additional support for altered functional properties of NOS in hypercholesterolemia was provided by findings showing that the supplementation of diet by L-arginine improved endothelium-mediated vasodilation in humans (*F98*) and reduced the formation of atherosclerotic lesions in hypercholesterolemic animals (*F99*).

Downregulation of NO synthesis has also been observed in macrophages exposed to oxidized LDL and subsequently activated by cytokines. Activated macrophages with oxidized LDL-induced downregulation of NO production, when exposed to native LDL, promoted significantly greater oxidative modification of LDL compared to either nonloaded macrophages or macrophages loaded with acetylated LDL (*F100*).

These observations would implicate the alterations in the production of NO as one of the mechanisms predisposing the endothelium to the development of foam cells and subsequent progression into atherosclerotic plaque. However, the answer to the key question, as to what is the determinant of increased formation of oxidatively modified LDL in hypercholesterolemia, awaits elucidation. One of the possible mechanisms may relate to the ability of endothelial cells and activated macrophages to generate superoxide radicals. In regenerated endothelium, the generation of superoxide is not impaired (*F101*). The normal capacity of endothelium to produce superoxide coupled with attenuated production of NO in the early phase of the atherosclerotic process may shift the physiological equilibrium toward relatively greater production of prooxidant species, including those generated by reaction of NO with superoxide (*F102–F104*), and result in propagation of pathological changes.

THERAPEUTIC APPLICATIONS OF MANIPULATION OF NO METABOLISM

Septic Shock. Hypotension, hyporesponsiveness to vasoconstrictors, and circulatory decompensation are characteristic features of septic shock. This clinical presentation is related to overproduction of NO as a result of upregulation of inducible NOS (*F1, F105, F106*). The attempts to correct abnormal hemodynamics in septic shock by inhibitors of NOS, either in animal models of septic shock (*F107–F110*) or in humans with septic shock refractory to treatment by vasoconstricting agents (*F111*), did not result in consistent outcomes. Although the administration of L-NMMA or L-NAME resulted in elevation of blood pressure, in some studies such an elevation was associated with underperfusion and ischemia.

The major drawback of using arginine analogues in the treatment of septic shock is their nonselectivity as these compounds inhibit both constitutive and inducible forms of NOS. Suppression of the constitutive form compromises the NO-mediated maintenance of basal tone in arterial circulation and permits unrestricted actions of endogenous vasoconstrictors on blood vessels. The resulting exacerbated increase in systemic resistance may progress into circulatory collapse. Another complication arises from the scarcity of pharmacokinetic data on the disposition of arginine analogues in the body. In an attempt to accommodate these shortcomings, it was shown that coadministration of L-NMMA with a NO donor capable of tonic release of low concentrations of NO (*S*-nitroso-*N*-acetylpenicillamine) in an animal model of septic shock offered significantly better outcomes as compared to administration of L-NMMA alone (*F108, F112*).

NO Inhalation. The efficacy of inhaled NO to achieve pulmonary vasodilation was initially tested in animal models of pulmonary hypertension induced by hypoxia or by infusion of a tromboxane analogue (*F113*). Inhalation of NO at dose of 5–80 ppm was shown to result in rapid reversal of pulmonary hypertension without causing hemodynamic changes in systemic circulation. This initial development was followed by an experimental study using a human model of pulmonary hypertension induced by inspiration of 12% oxygen. Inhalation of NO (40 ppm) reversed hypoxia-induced pulmonary vasoconstriction without altering systemic vascular resistance or arterial blood pressure (*F114*). In control subjects, inhaled NO did not elicit significant changes in pulmonary or systemic circulation.

These studies provided evidence that the inhalation of low doses of NO may serve as a safe and efficient way for selective delivery of this vasoactive agent into pulmonary blood vessels without untoward effects due to potential formation of toxic products (such as higher oxides of nitrogen). Furthermore, efficient scavenging of NO by hemoglobin after diffusion into blood (reflected in slight elevation of methemoglobin levels during NO inhalation) prevents the development of vasodilation in systemic circulation. The beneficial effects of NO inhalation were confirmed in several clinical case studies which applied this treatment to patients with pulmonary hypertension associated with various respiratory disorders, including severe adult respiratory syndrome (*F115*), persistent pulmonary hypertension of the newborn (*F116*), primary pulmonary hypertension (*F117*) and bacteremic pneumococcal pneumonia (*F118*).

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