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# Nitric oxide as a mediator of oxidant lung injury due to paraquat

(neurotransmitters/oxidant tissue injury/free radicals)

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ABSTRACT **At low concentrations, nitric oxide is a physiological transmitter, but in excessive concentrations it may cause cell and tissue injury. We report that in acute oxidant injury induced by the herbicide paraquat in isolated guinea pig lungs, nitric oxide synthesis was markedly stimulated, as evidenced by increased levels of cyclic GMP in lung perfusate and of nitrite and L-citrulline production in lung tissue. All signs of injury, including increased airway and perfusion pressures, pulmonary edema, and protein leakage into the airspaces, were dose-dependently attenuated or totally prevented by either $N^G$-nitro-L-arginine methyl ester or $N^\omega$-nitro-L-arginine, selective and competitive inhibitors of nitric oxide synthase. Protection was reversed by excess L-arginine but not by its enantiomer D-arginine. When blood was added to the lung perfusate, the paraquat injury was moderated or delayed as it was when paraquat was given to anesthetized guinea pigs. The rapid onset of injury and its failure to occur in the absence of $Ca^{2+}$ suggest that constitutive rather than inducible nitric oxide synthase was responsible for the stimulated nitric oxide synthesis. The findings indicate that nitric oxide plays a critical role in the production of lung tissue injury due to paraquat, and it may be a pathogenetic factor in other forms of oxidant tissue injury.**

Now widely recognized as a physiological transmitter of diverse and vital functions (1, 2), nitric oxide ($NO^·$), if excessively produced, can cause cellular and chromosomal damage (3). There is evidence that $NO^·$ can contribute to glutamate neurotoxicity (4), the persistent hypotension of septic shock (5–8), autoimmune destruction of pancreatic islet cells (9), and immune complex-induced injury (10). How and under what conditions $NO^·$ may mediate tissue injury remain open questions. One possible mechanism is for $NO^·$ to react with the superoxide radical, also generated by the action of $NO^·$ synthase (11, 12), to form peroxynitrites (13, 14). We have now examined whether $NO^·$, itself a free radical, may be a pathogenetic factor in acute oxidant tissue injury. We report that lung tissue injury believed to result from reactive oxygen species (15–17) generated by the herbicide paraquat was associated with augmented $NO^·$ synthesis, was largely or totally prevented by inhibitors of this synthesis, and was moderated in the presence of hemoglobin. The results support the view that $NO^·$ is a critical intermediary in the production of oxidant tissue damage due to paraquat, and possibly other prooxidants, and suggest means of defense against acute oxidant stress.

## MATERIALS AND METHODS

**Isolated Lung Preparation.** Guinea pigs (Hartley strain, male; 450–550 g) were anesthetized with intraperitoneal injection of sodium pentobarbital (100 mg/kg) and intubated intratracheally. The lungs were ventilated with a rodent respirator (model 640; Harvard Apparatus) at 40–50 cycles per min and a tidal vol of 6 ml/kg, with a humidified gas mixture of 95% $O_2$/5% $CO_2$. (We used 95% $O_2$ because oxidant injury is enhanced at higher $O_2$ tensions.) A positive end-expiratory pressure of 2 cm of $H_2O$ was applied to reduce atelectasis. The chest was opened and the heart was exposed. After an injection of heparin (400 USP units) into the inferior vena cava, an outflow cannula was placed in the left atrium through an incision in the left ventricle, and an inflow cannula was inserted into the main pulmonary artery (PA) through the right ventricle. The lungs were perfused with Krebs solution (117.6 mM NaCl/5.4 mM KCl/1.2 mM $MgSO_4$/25.0 mM $NaHCO_3$/1.0 mM $NaH_2PO_4$/2.5 mM $CaCl_2$) containing 4% bovine serum albumin (Krebs-BSA) and equilibrated with 95% $O_2$/5% $CO_2$. Perfusion was started at a rate of 9 ml/min by a pulsatile circulation pump (model 1405; Harvard Apparatus) and the rate was adjusted to achieve an initial mean perfusion pressure ($P_{PA}$) of 5–8 cm of $H_2O$ and was then held constant. Initially, the lungs were perfused in an open, nonrecirculating mode for 10 min to wash out residual blood, after which perfusion was changed to a recirculating mode with 120 ml of Krebs-BSA. The lung preparation was allowed to equilibrate for 10 min under constant perfusion and ventilation before any intervention was begun.

In this preparation, with the lungs perfused at a constant flow and mechanically ventilated at a constant rate and breath volume, acute lung injury, leading to high-permeability pulmonary edema, is marked by sharp increases in the peak airway pressure ($P_{AW}$), in the $P_{PA}$, and in the wet weight of the lung, as well as by leakage of protein into the airspaces (18, 19).

**Physiological Measurements.** Peak $P_{AW}$ and $P_{PA}$ were continuously monitored by pressure transducers (Statham P23A; Statham Instruments, Hato Rey, PR) attached to the tracheal cannula and to a polyethylene catheter in the main PA, respectively, and were recorded (2600S recorder; Gould, Cleveland). Left atrial pressure was maintained at 2 cm of $H_2O$ by placing the tip of the outflow cannula 2 cm above the level of the atrium. To assess the contribution of hydrostatic forces to the induced pulmonary edema, we periodically measured pulmonary microvascular pressure by the double-occlusion method (20). At the end of the experiment, the left lung was lavaged with 3 ml of saline for measurement of bronchoalveolar lavage (BAL) fluid protein content, as an index of protein leakage due to alveolar–microvascular membrane injury. The right lung was dissected free of major airways and connective tissue, gently blotted, and then weighed (wet weight) and oven-dried to a constant weight to determine dry weight and wet/dry (W/D) lung weight ratio as measures of the severity of pulmonary edema.

**Induction of Lung Injury.** Acute lung injury was produced by paraquat (100 mg/kg of the dichloride salt, dissolved in 1 ml of 0.9% NaCl), which was infused directly into the

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Abbreviations: PA, pulmonary artery; AW, airway; BAL, bronchoalveolar lavage; W/D, wet/dry lung weight ratio; L-NAME, $N^G$-nitro-L-arginine methyl ester.

pulmonary artery of guinea pig lungs over 1 min by an infusion pump (model 940-S; Harvard Apparatus). Perfusion was continued until pulmonary edema occurred, as marked by the appearance of foam in the airway, or for 60 min.

In four separate experiments designed to assess the effect of hemoglobin on paraquat injury, the lungs were perfused with Krebs-BSA to which was added heparin-treated blood from the same animal to a final hemoglobin concentration of 1–2 g/dl. In three additional experiments, paraquat was infused intravenously into guinea pigs anesthetized with ketamine/xylazine and mechanically ventilated via an endotracheal tube with 95% $O_2$/5% $CO_2$. In these animals, we monitored airway pressure and arterial blood pressure and, at the end of the experiment, measured lung weights and BAL protein content as described above.

**Inhibitors of NO· Synthase.** We evaluated the influence of the NO· synthase inhibitors $N^G$-nitro-L-arginine methyl ester (L-NAME; 2 mM) and $N^\omega$-nitro-L-arginine (nitroarginine; 2 or 10 mM) (1) on the occurrence and severity of lung injury and the increased production of NO·. The inhibitors were added beginning 10 min before the administration of paraquat and continued for the duration of the experiment. The specificity of the responses to the inhibitors was confirmed by testing their reversibility with excess (10 mM) L-arginine ($n$ = 5) or its enantiomer D-arginine ($n$ = 4) present in the perfusate.

**Requirement for $Ca^{2+}$.** To determine whether the increased NO· production was caused by activation of the $Ca^{2+}$-dependent constitutive NO· synthase, or the inducible $Ca^{2+}$-independent enzyme, we attempted to produce the paraquat injury under the same conditions but in the absence of $Ca^{2+}$. To this end, guinea pig lungs were perfused with Krebs solution modified such that $CaCl_2$ was replaced by an equimolar concentration of $MgCl_2$, and paraquat was given as described above ($n$ = 3).

**Assay of NO· Synthase.** *cGMP levels.* As an index of NO· production, cGMP levels were measured in lung perfusate by a specific RIA in the presence of the phosphodiesterase inhibitor isobutylmethylxanthine (1 mM). $^{125}$I-labeled cGMP was diluted to provide 7000 cpm per 100 μl, and antibody was diluted to yield a maximal binding of 40–50% of total counts. Samples were usually acetylated to enhance the sensitivity of the assay. Antigen–antibody complexes were precipitated with excess rabbit nonimmune immunoglobulin G and polyethylene glycol, achieving a recovery of cGMP (>95%). The concentration of cGMP was expressed per ml of lung perfusate or per mg of protein of lung tissue.

*L-Citrulline production.* The formation of $^{14}$C-labeled L-citrulline, measured as an index of NO· production from L-arginine, was performed as described (21). Lung tissue cytosol preparations were incubated for up to 60 min with [$^{14}$C]L-arginine and cofactors, as for determination of nitrite levels. Measurements were also made on control samples in which $CaCl_2$ was omitted and EGTA (1 mM) was added, L-NAME (100 μM) was added, or Tris·HCl buffer replaced cytosol. $^{14}$C-labeled L-citrulline production (corrected for values in blank samples) was expressed per mg of protein in lung tissue cytosol.

*Nitrite ($NO_2^-$) and nitrate ($NO_3^-$) levels.* Cytosol fractions were prepared (22, 23) from lung tissue samples taken before and after acute injury and incubated for 45 min at 37°C in Tris·HCl buffer (pH 7.8) in the presence of $CaCl_2$ (2 mM), tetrahydrobiopterin (10 μM), FAD (10 μM), NADPH (1 mM), and L-arginine (1 mM). The reaction was terminated and the stable end products of NO·, $NO_2^-$, and $NO_3^-$ were assayed spectrophotometrically with the Griess reagent as described (24). Nonspecific absorbance was measured in the absence of L-arginine and NADPH and was subtracted from that of the test samples. $NO_2^-$ content was expressed per mg of protein in lung tissue cytosol.

**Experimental Groups.** In the isolated lung model with Krebs-BSA perfusion, there were eight experimental groups: paraquat only, $n$ = 13; paraquat + L-NAME, $n$ = 6; paraquat + L-NAME + L-arginine, $n$ = 5; paraquat + L-NAME + D-arginine, $n$ = 4; paraquat + 2 mM nitroarginine, $n$ = 3; paraquat + 10 mM nitroarginine, $n$ = 3; paraquat in $Ca^{2+}$-free perfusate, $n$ = 3; saline control group, $n$ = 7.

**Reagents.** The cGMP kit was from New England Nuclear/DuPont; all other chemicals, including paraquat (methyl viologen), L-NAME, nitroarginine, and superoxide dismutase (EC 1.15.1.1; from bovine liver) were from Sigma.

**Statistical Analysis.** The influence of each intervention on functional and biochemical measurements was assessed by analysis of variance followed by Tukey's procedures for intergroup comparison (25). Repeated measurements of PAW and PPA over time were analyzed by multiple paired $t$ tests.

## RESULTS

**Lung Injury due to Paraquat.** *In lungs perfused with Krebs-BSA.* Within 20 sec of the infusion of paraquat (100 mg/kg) into the PA, PAW increased rapidly from a baseline value of 10.4 ± 0.6, reaching a peak of 51.0 ± 8.4 cm of $H_2O$ at ≈3 min, a 390% increase (Fig. 1*A*). After this initial sharp increase, PAW declined to 33.0 ± 7.4 at 15 min but remained elevated for the duration of the experiment ($n$ = 13; $P < 0.01$). PPA also increased but more moderately, from 7.3 ± 0.5 to a peak of 15.8 ± 2.9 cm of $H_2O$ at 5 min (116% increase), then declined to 13.9 ± 2.4 at 15 min (Fig. 1*B*), and remained approximately at that level for the rest of the experiment ($n$ = 13; $P < 0.01$). Control microvascular pressure, measured in six experiments by the double-occlusion method, was 3.0 ± 0.13 cm of $H_2O$ and did not change significantly with paraquat infusion. Mean W/D weight ratio of paraquat-treated lungs was 7.02 ± 0.31 ($n$ = 13), 34% higher than for lungs of the control group, which had a mean ratio of 5.23 ± 0.03 ($P < 0.01$; Fig. 1*C*). The protein content of BAL fluid from paraquat-treated lungs ($n$ = 12) was 2.53 ± 0.59 mg/ml, a 278% increase from the 0.67 ± 0.20 mg/ml in the control group ($P < 0.01$; Fig. 1*D*).

*In lungs perfused with blood-containing buffer.* In these experiments, evidence of injury was observed but it was generally less severe; at the end of 1 h of perfusion, PAW was 23 ± 2.2, PPA was 9 ± 1.5, W/D was 5.97 ± 0.44, and BAL protein was 0.86 ± 0.40.

*In whole animals.* In two of the three experiments, PAW increased to twice or 3.8 times the control value and mean arterial blood pressure decreased to near zero at 60 and 105 min, respectively, after the injection of paraquat. In both of these animals the lungs were grossly edematous (W/D = 7.17), with moderately increased BAL protein content (0.48 mg/ml). In the third animal, the experiment was terminated at 1 h, at which time PAW had increased by only 17%, but blood pressure and W/D were at basal values.

**Attenuation of Injury by NO· Synthase Inhibitors.** The addition of either of the two NO· synthase inhibitors, L-NAME (2 mM) or nitroarginine (2 or 10 mM), to the perfusate markedly attenuated or totally prevented all signs of injury. In the presence of L-NAME ($n$ = 6), PAW increased moderately from a baseline value of 10.5 ± 0.5 to 16.3 ± 3.7 cm of $H_2O$ 1 min after paraquat, quickly returned toward the baseline, and was normal (11.3 ± 0.7 cm of $H_2O$) at 60 min. PPA stayed at basal value through the 60-min observation period. W/D was 5.61 ± 0.14, 22% lower than in the paraquat group ($P < 0.01$), and BAL protein content was 0.45 ± 0.1, 83% lower than in paraquat-treated lungs ($P < 0.01$); both were no different from values in uninjured lungs. When excess (10 mM) L-arginine was added to the perfusate together with L-NAME ($n$ = 5), to confirm the specificity of the protective effect of L-NAME, lung injury was again in

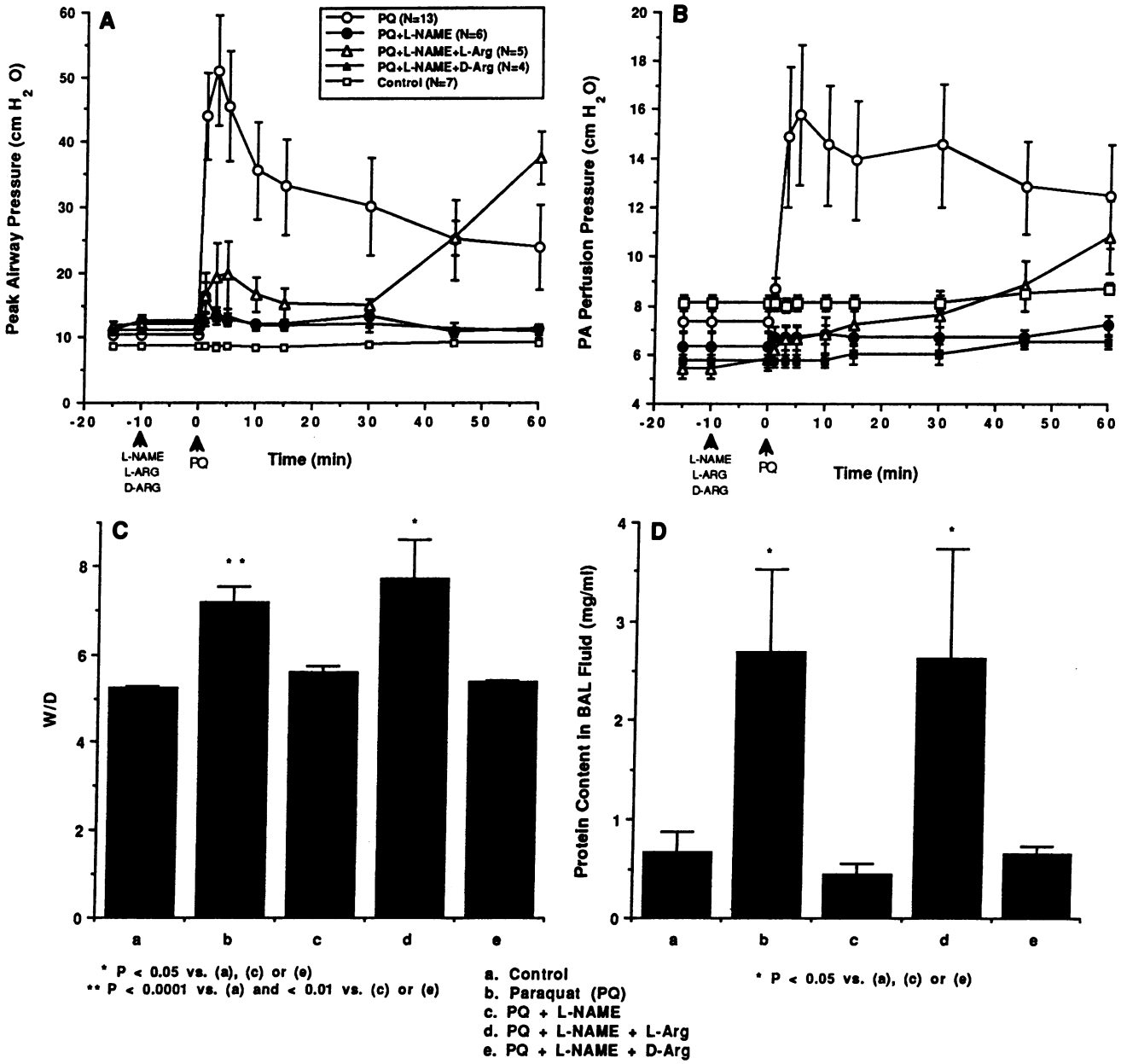


FIG. 1. Paraquat-induced increases in peak PAW (*A*), mean PPA (*B*), W/D (*C*), and protein content (mg/ml) in BAL fluid, obtained by lavaging the left lung with 3 ml of saline at the end of the experiment (*D*). All variables are plotted before and after a 1-min infusion of paraquat (100 mg/kg) into the perfusate. The lungs were mechanically ventilated with 95% $O_2$/5% $CO_2$ and perfused *in situ* at 30 ml/kg·min$^{-1}$ with Krebs solution containing 4% BSA at 37°C. All increases induced by paraquat were totally prevented by an infusion of 2 mM L-NAME to the perfusing medium, beginning 10 min before paraquat ($P < 0.01$). Excess L-arginine reversed the L-NAME effect ($P < 0.01$), but D-arginine did not.

evidence: PAW increased markedly 45 min after the administration of paraquat, from a baseline value of 11.8 ± 0.7, reaching 37.4 ± 4.1 at 60 min; PPA increased to 10.8 ± 1.5 from a baseline of 5.4 ± 0.4; W/D was 7.72 ± 0.88; and BAL protein content was 2.63 ± 1.1 mg/ml, both significantly higher than in the L-NAME + paraquat group ($P < 0.01$) and as high as in the paraquat group. L-Arginine alone had no effect, and D-arginine in the same concentration ($n = 4$) did not alter the inhibition by L-NAME (Fig. 1 *C* and *D*).

In the presence of 2 mM nitroarginine, PAW increased only moderately and transiently, from a baseline of 10.0 ± 0.0 to a peak of 14.0 ± 2.0 cm of $H_2O$ after 60 min; PPA remained unchanged at 6.31 ± 0.31; W/D was slightly increased at 5.70 ± 0.1; and BAL protein content was 0.49 ± 0.01 mg/ml. At 10 mM nitroarginine, all signs of injury were abolished: PAW and PPA remained at or close to baseline values of 9.0 ± 0.57 and 6.3 ± 0.33 cm of $H_2O$, respectively, after 60 min of infusion with paraquat. W/D decreased further to a normal 4.72 ± 0.04 ($P < 0.002$, compared with 2 mM nitroarginine; Fig. 2), and protein content in BAL fluid was a low 0.26 ± 0.05 ($P < 0.01$ compared with 2 mM nitroarginine).

**Evidence for Increased NO· Production.** cGMP levels in perfusate effluent, measured as an index of NO·-dependent guanylyl cyclase activation, increased up to 6-fold with the onset of injury (from 0.53 ± 0.15 to 2.88 ± 0.0 pmol/ml; $P <$ 0.05 vs. baseline value) but did not increase in lungs treated with L-NAME together with paraquat ($n = 4$ for each group, $P < 0.005$ between corresponding points in each group; Fig. 3).

**L-Citrulline Production.** $^{14}$C-labeled L-citrulline production increased markedly in cytosol fractions of lungs treated with paraquat, reaching a mean of 45,598.2 ± 1105.2 dpm per mg of protein ($P < 0.001$; $n = 4$). The increase was progressive over a 60-min period but was sharpest in the first 5 min. In the presence of EGTA or L-NAME, the corresponding values

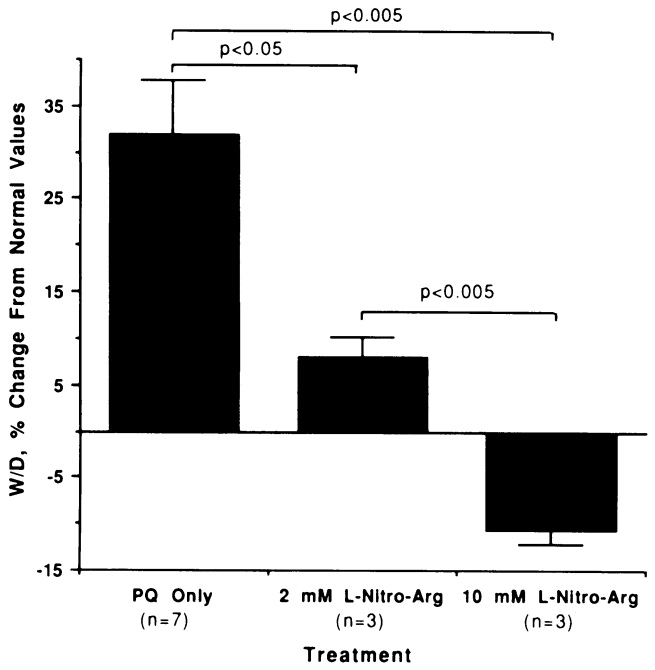


FIG. 2. Dose-dependent attenuation of paraquat (PQ)-induced increases in W/D by 2 and 10 mM L-nitroarginine.

were reduced to 5694.8 ± 1313.4 and 6082.8 ± 1058.2 dpm per mg of protein, respectively ($n$ = 4).

**Nitrite/Nitrate Accumulation.** Stimulated NO· production was further confirmed in two paraquat-treated lungs where $NO_2^-/NO_3^-$ levels in lung tissue cytosol increased to 27.4 and 68.3 μmol per mg of protein from a baseline value of 18.2 μmol per mg of protein.

**$Ca^{2+}$ Dependence of Injury.** When paraquat was given to guinea pig lungs perfused with $Ca^{2+}$-free Krebs-BSA ($n$ = 3), PAW increased only slightly, from 7.6 ± 0.31 to 11.3 ± 2.3 cm of $H_2O$; PPA, W/D, and BAL protein content were the same as in normal control lungs and significantly lower ($P < 0.0007$) than corresponding values in the presence of $Ca^{2+}$.

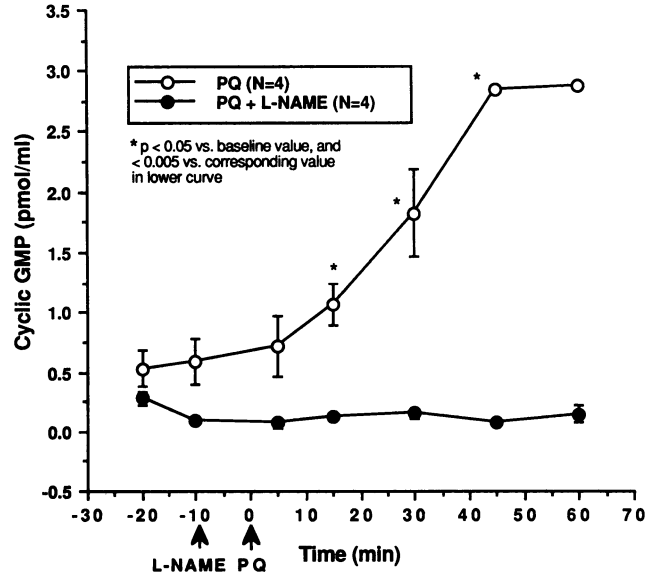


FIG. 3. cGMP levels in lung perfusate began to increase within 15 min of addition of paraquat (PQ) and continued to increase for the balance of the experiment, reaching a plateau around 45 min. In the presence of L-NAME, the levels were reduced from the beginning and remained low throughout.

## DISCUSSION

The findings presented here demonstrate that the acute oxidant tissue injury of guinea pig lung induced by paraquat was markedly attenuated or totally prevented by either of two selective and competitive inhibitors of NO· synthase, L-NAME or nitroarginine. The injury was associated with increased production of cGMP that was reversible with L-NAME and of L-citrulline and nitrite levels in lung cytosol. Furthermore, the protection against lung injury by NO· synthase inhibitors was partially or completely reversible in the presence of excess L-arginine, but not its enantiomer D-arginine. The lung injury was moderated in the presence of low concentrations of hemoglobin in the lung perfusate, or when paraquat was infused into guinea pigs *in vivo*. Taken together, these results strongly suggest that paraquat stimulated NO· synthesis, and that the excess NO· produced was a key factor in the induction of tissue injury.

The high affinity of NO· for hemoglobin was first reported as far back as 1865 (26) and later confirmed and quantified (27). More recently, several authors have documented the loss of the vasodilator activity of NO· in the presence of hemoglobin (28). Our results show that the toxic effects of NO· were attenuated when hemoglobin was added to the perfusate of the isolated lung or when paraquat was infused in whole guinea pigs. The scavenging of NO· by hemoglobin may also explain why pulmonary injury from the ingestion of paraquat in humans, or even its intravenous injection in some animal species, including rats, has a latent period of at least several hours (15). Guinea pigs are more susceptible than rats to paraquat toxicity, especially when associated with high inspired oxygen tensions, as in our experiments.

The mechanisms by which NO· may induce tissue injury are not fully understood, but they include the generation and interaction with other toxic free radicals. The precise contribution of reactive oxygen metabolites, until now thought to be the sole mediators in paraquat lung injury (15–17, 19, 20), should now be reexamined and their relationship to the induction of NO· synthesis defined. Among these radicals are the superoxide radical, which is coproduced with NO· (11, 12) and interacts with it to form the peroxynitrite anion (13, 14). The latter decomposes to hydroxyl radical (11) and nitrogen dioxide (13). In addition, NO· has recently been shown to react with hydrogen peroxide to produce singlet oxygen (29), a highly reactive oxygen species. Superoxide dismutase, which scavenges superoxide, attenuates *N*-methyl-D-aspartate toxicity (12, 30). Superoxide dismutase, however, was ineffective against $O_2$ toxicity of the central nervous system (30) or oxidant lung injury due to xanthine and xanthine oxidase (31), possibly because its stabilization of NO· (32) in those models enhances the toxicity.

Another question is which NO· synthase was responsible for producing the excess NO· that led to lung injury. The onset of lung injury in these experiments occurred within 5 min, and the injury was fully in evidence within 1 h. The short time required for the injury to develop, coupled with its dependence on the presence of $Ca^{2+}$ in the perfusate, suggests that the stimulated NO· output was generated by activation of the $Ca^{2+}$-dependent constitutive enzyme, in neuronal or endothelial cells, rather than to induction of the inducible $Ca^{2+}$-independent enzyme prevalent in macrophages (1). This conclusion is supported by the recent observation from dot-blot hybridization studies that paraquat increases mRNA levels of neuronal NO· synthase in pulmonary tissue (A. Bandyopadhyay, S. Rattan, and S.I.S., unpublished data). Thus, NO·-mediated paraquat lung injury seems analogous to the rapidly triggered, glutamate-induced killing of cortical neuronal cells in which NO· may play an essential role (4). The dynamics of both forms of tissue injury imply that toxic levels of NO· may be produced not only by

induction of the inducible NO· synthase, as by endotoxin lipopolysaccharide and cytokines (6, 7, 22, 33), but also by up-regulation of the constitutive enzyme. The latter enzyme has commonly been viewed merely as a source of physiological NO· acting as a neurotransmitter (2) or as endothelium-derived relaxant factor (34, 35).

Oxidative stress and excessive NO· production have already been linked to neuronal cell toxicity and neurodegenerative disorders (36–38). The present data provide evidence for a similar link with lung tissue injury. The lung injury in the paraquat model tested here resembles in some respects that seen in the adult respiratory distress syndrome and multiorgan failure, which affects >200,000 subjects per year in the United States, with a mortality rate of >60% (39). Identification of NO· as a critical intermediate in the events leading to this injury, if confirmed in patients with the clinical disorder, should lead to additional approaches toward reducing its incidence and mortality.

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