Am J Physiol Lung Cell Mol Physiol
280: L326–L333, 2001.

Toll-like receptor 4 mediates ozone-induced murine lung hyperpermeability via inducible nitric oxide synthase

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Received 26 May 2000; accepted in final form 28 September 2000

Kleeberger, Steven R., Sekhar P. M. Reddy, Liu-Yi Zhang, Hye-Youn Cho, and Anne E. Jedlicka. Toll-like receptor 4 mediates ozone-induced murine lung hyperpermeability via inducible nitric oxide synthase. *Am J Physiol Lung Cell Mol Physiol* 280: L326–L333, 2001.—We tested the hypotheses that *1*) inducible nitric oxide synthase (iNOS) mediates ozone (O_3)-induced lung hyperpermeability and *2*) mRNA levels of the gene for iNOS (*Nos2*) are modulated by Toll-like receptor 4 (*Tlr4*) during O_3 exposure. Pretreatment of O_3-susceptible C57BL/6J mice with a specific inhibitor of total NOS (N^G-monomethyl-L-arginine) significantly decreased the mean lavageable protein concentration (a marker of lung permeability) induced by O_3 (0.3 parts/million for 72 h) compared with vehicle control mice. Furthermore, lavageable protein in C57BL/B6 mice with targeted disruption of *Nos2* [*Nos2*(−/−)] was 50% less than the protein in wild-type [*Nos2*(+/+)] mice after O_3. To determine whether *Tlr4* modulates *Nos2* mRNA levels, we studied C3H/HeJ (HeJ) and C3H/HeOuJ mice that differ only at a missense mutation in *Tlr4* that confers resistance to O_3-induced lung hyperpermeability in the HeJ strain. *Nos2* and *Tlr4* mRNA levels were significantly reduced and correlated in resistant HeJ mice after O_3 relative to those in susceptible C3H/HeOuJ mice. Together, the results are consistent with an important role for iNOS in O_3-induced lung hyperpermeability and suggest that *Nos2* mRNA levels are mediated through *Tlr4*.

innate immunity; epithelium; inflammation; polymorphism; knockout mouse

IN HEALTHY HUMAN SUBJECTS, ozone (O_3) causes decrements in pulmonary function and induces airway inflammation and hyperpermeability. Although O_3 continues to be a major public health concern (1, 9, 17, 31, 48), the mechanisms of injury and associated susceptibility factors are not completely understood. Kleeberger et al. (23) previously demonstrated that susceptibility to lung hyperpermeability induced by subacute O_3 exposure is a quantitative trait in inbred mice. Genetic linkage analyses with O_3-susceptible C57BL/6J (B6) and O_3-resistant C3H/HeJ (HeJ) mice identified significant and suggestive quantitative trait loci for susceptibility to O_3-induced hyperpermeability on chromosomes 4 and 11, respectively. Within the chromosome 4 quantitative trait locus, Toll-like receptor 4 (*Tlr4*) was identified as a candidate gene for differential O_3 susceptibility. *Tlr4* has been identified as the gene that determines susceptibility to endotoxin challenge in mice (6, 40, 42). Strong supportive evidence for this gene candidate in O_3 susceptibility was provided by two observations. First, O_3-induced lung permeability was significantly different between HeJ and C3H/HeOuJ (OuJ) mice, which differ only at a missense mutation in the third exon of the *Tlr4* gene (40). Second, *Tlr4* mRNA levels in the lungs of HeJ mice were decreased relative to those in OuJ mice after exposure to O_3, which suggested that downregulation of *Tlr4* gene expression may contribute to O_3 resistance in HeJ mice.

The mechanism(s) through which *Tlr4* mediates differential O_3-induced hyperpermeability is not understood. Nitric oxide (NO) has received considerable attention over the last decade for its homeostatic and "protective" roles as well as for its pathological and "injurious" roles in many tissue and organ systems. NO is produced from L-arginine with citrulline (a by-product) by nitric oxide synthase (NOS) activity in many tissue types. Three isoforms of NOS have been identified and the genes for each have been cloned. Endothelial NOS and neuronal NOS are constitutive forms that were initially characterized in endothelial and neuronal cells, respectively (10). Endothelial NOS has an important role in the maintenance of vascular tone, whereas neuronal NOS is a neurotransmitter that activates soluble guanylate cyclase and increases intracellular cGMP (see Ref. 10 for review). An inducible form of NOS, iNOS, was initially characterized in murine macrophages and has subsequently been identified in many cell types. In phagocytic cells of mice and other species, induction of iNOS results in prolonged production of NO and is a primary mechanism in the phagocytic killing of viruses, bacteria, and tumor cells (5, 10, 34). For example, iNOS-derived NO has antimicrobial properties during infection with *Leishmania major* (3) and *Klebsiella pneumoniae* (49). Inhaled NO has also been shown to enhance ventilation and perfu-

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sion matching in subgroups of patients with acute respiratory distress syndrome (28, 43).

NO may also have detrimental effects by directly inducing tissue damage (30, 52), leading to compromised epithelial integrity and increased epithelial permeability (13, 50, 51). In particular, a role for NO in the endotoxin-induced change in acute lung injury (29, 32), vascular tone (12, 47), and alveolar epithelial permeability (32) has been demonstrated. Furthermore, Pendino and colleagues and Punjabi et al. have shown that 3 h of exposure to 2 parts/million (ppm) O_3 causes NO production in macrophages (37–39) and type II cells (41) of rats, whereas Haddad et al. (14) demonstrated iNOS induction in rats after 6 h of exposure to 3 ppm O_3.

Because *Tlr4* mediates lung responses to endotoxin and O_3 and iNOS contributes to endotoxin-induced lung hyperpermeability, we hypothesized that iNOS similarly mediates a lung permeability change induced by subacute O_3 exposure. There were two objectives of this study. The first was to determine the role of iNOS in the pathogenesis of O_3-induced lung hyperpermeability in mice. To initially address this objective, we evaluated the effect of N^G-monomethyl-L-arginine (L-NMMA), an inhibitor of total NOS, on O_3-induced hyperpermeability in B6 mice. We also evaluated the specific role of iNOS in B6 mice with a site-directed mutation (knockout) of the gene for iNOS [*Nos2*(−/−)]. *Nos2*(−/−) mice were exposed to O_3, and the hyperpermeability response was compared with that in wild-type [*Nos2*(+/+)] control mice. The second objective of this study was to test the hypothesis that O_3-induced *Nos2* mRNA levels are regulated by *Tlr4*. *Nos2* mRNA transcripts in lung homogenates from O_3-exposed HeJ and OuJ mice were amplified by reverse transcriptase-polymerase chain reaction, quantitated, and compared between strains. Results of these experiments are consistent with a strong role for iNOS in O_3-induced lung hyperpermeability and suggest that *Tlr4* is an important determinant of *Nos2* expression.

METHODS

General. Male (6- to 8-wk-old, 20- to 25-g) inbred strains of mice {HeJ, OuJ, *Nos2*(+/+) [C57BL/6J-*Nos2*(+/+)], and *Nos2*(−/−) [C57BL/6J-*Nos2*(−/−)]} were purchased from Jackson Laboratories (Bar Harbor, ME). All animals were housed in a virus- and antigen-free room. Water and mouse chow were provided ad libitum. Cages were placed in laminar flow hoods with high-efficiency particulate-filtered air. Sentinel animals were examined periodically (titers and necropsy) to ensure that the animals had remained free of infection. All experimental protocols conducted in the mice were carried out in accordance with the standards established by the US Animal Welfare Acts and set forth in National Institutes of Health guidelines and the *Policy and Procedures Manual* (Johns Hopkins University School of Hygiene and Public Health, Baltimore, MD, Animal Care and Use Committee).

O_3 generation and exposure. Mice were placed individually in stainless steel wire cages with free access to food and water during the exposure. The cages were set inside one of two separate 700-liter laminar flow inhalation chambers (Baker, Sanford, ME) that were equipped with a charcoal- and high-efficiency particulate-filtered air supply. Chamber air was renewed at the rate of ~20 changes/h, with 50–65% relative humidity and a temperature of 20–25°C. O_3 was generated by directing air (2 l/min) through an ultraviolet light O_3 generator (Orec, Phoenix, AZ) that was upstream from one of the exposure chambers. The O_3-air mixture was metered into the inlet airstream with computer-operated stainless steel mass flow controllers. O_3 concentrations were monitored regularly at different levels within the chamber with an O_3 ultraviolet light photometer (Dasibi model 1003 AH, Dasibi Environmental, Glendale, CA). The Dasibi model 1003 AH was calibrated regularly against a standard source (Dasibi model 1008-PC, Dasibi Environmental). Simultaneous exposures to filtered air were done in age- and strain-matched mice to serve as O_3 controls.

Lung lavage and cell preparation. Bronchoalveolar lavage (BAL) was performed immediately after O_3 exposure. The mice were killed by cervical dislocation, and the lungs were lavaged in situ four times with Hanks' balanced salt solution (35 ml/kg; pH 7.2–7.4). In the gene expression experiments, a suture was tied around the left main stem bronchus of each mouse, and the lung was removed distal to the suture and immediately frozen in liquid nitrogen. The right lung was then lavaged (17 ml/kg) as described above. Recovered BAL fluid was immediately cooled to 4°C and centrifuged. The cell pellets were resuspended in 1 ml of Hanks' balanced salt solution, and the cells were counted with a hemacytometer. Aliquots (10 μl) were cytocentrifuged, and the cells were stained with Diff-Quik for differential cell analysis. The supernatants were assayed for total protein (a marker of lung permeability) by the Bradford (4) assay.

Nos2 and Tlr4 mRNA levels. Total RNA was isolated from lung tissues of HeJ and OuJ mice by homogenization in TRIzol reagent (Life Technologies) following the manufacturer's recommended protocol. cDNA was prepared by reverse transcribing 5 μg of total RNA primed with oligo(dT) with the SuperScript preamplification system (Life Technologies). Amplification was done under the following conditions: 1.5 mM $MgCl_2$, 70°C annealing temperature, extension time of 1 (*Nos2*) or 2.5 (*Tlr4*) min, and 30 cycles. Primers for *Nos2* and *Tlr4* were synthesized according to Kleinert et al. (24) and Poltorak et al. (40), respectively. β-Actin was simultaneously amplified as an internal (reference) control. Fragments were analyzed on 1.3% agarose gels. The amplified cDNA fragments were scanned and quantitated with a Bio-Rad Gel Doc 2000 system (Bio-Rad Laboratories, Hercules CA).

Statistics. Statistical analyses of the O_3-induced change in lung permeability were done by ANOVA (SuperANOVA statistical package, Abacus Concepts, Berkeley, CA). The Student-Newman-Keuls a posteriori test was used for comparisons of means (45). Statistical analyses of the relative changes in mRNA levels were done by nonparametric Kruskall-Wallis two-factor ANOVA (45). The factors were strain (HeJ and OuJ) and time (0 and 90 min and 3, 6, 24, 48, and 72 h). Significance for all comparisons of means was accepted at $P < 0.05$.

RESULTS

Role of NOS in O_3-induced increase in lung permeability. The role of NOS in the O_3-induced increase in lung permeability was assessed by treating B6 mice with L-NMMA (2.1 mg/mouse ip; Sigma) immediately before exposure to O_3 or filtered air was begun. A second, boosting dose (1 mg/mouse ip) was administered after 24 h of exposure. Compared with that in

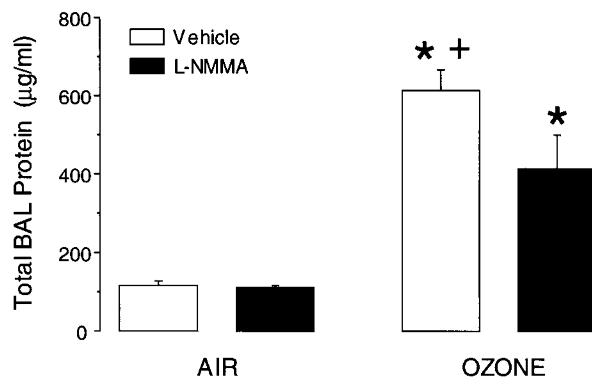

Fig. 1. Inhibition of nitric oxide synthase with N^{G}-monomethyl-L-arginine (L-NMMA) significantly attenuated the change in total bronchoalveolar lavage (BAL) fluid protein induced by 72 h of exposure to 0.3 parts/million (ppm) ozone (O_3) in C57BL/6J mice (n = 5–8/group). Vehicle was Hanks' balanced salt solution. $^{*}P < 0.05$ vs. air. $^{+}P < 0.05$ vs. L-NMMA.

vehicle-treated mice, L-NMMA did not significantly affect total BAL fluid protein in mice exposed to air for 72 h (Fig. 1). However, the mean BAL fluid protein concentration in L-NMMA-treated mice exposed to O_3 for 72 h (399 ± 85 μg/ml) was significantly attenuated by 35% relative to that in O_3-exposed vehicle control mice (615 ± 52 μg/ml; $P < 0.05$; Fig. 1).

No significant differences in the mean number of lavageable macrophages, polymorphonuclear leukocytes (PMNs), or epithelial cells were found between L-NMMA- and vehicle-treated mice exposed to filtered air (Table 1). Relative to air control mice, O_3 caused significant increases in each of the cell types in L-NMMA- and vehicle-treated mice ($P < 0.05$; Table 1). No significant differences in the mean number of macrophages, PMNs, or epithelial cells were found between L-NMMA- and vehicle-treated mice exposed to O_3 (Table 1).

Wild-type mice [C57BL/6J-*Nos2*(+/+)] and mice with a site-directed mutation (knockout) of *Nos2* [C57BL/6J-*Nos2*(−/−)] were exposed to O_3 and filtered air to determine the contribution of iNOS to O_3-induced lung hyperpermeability. Mean BAL fluid protein concentrations of air-exposed *Nos2*(+/+) (100 ± 13 μg/ml) and *Nos2*(−/−) (89 ± 6 μg/ml) mice were not significantly different from each other (Fig. 2). Relative to the respective air-exposed control mice, O_3 caused significant increases in the mean BAL fluid protein in *Nos2*(+/+) and *Nos2*(−/−) mice after 48 and 72 h of exposure ($P < 0.05$; Fig. 2). However, compared with that in wild-type *Nos2*(+/+) mice, O_3-induced BAL fluid protein was significantly reduced by 36% in *Nos2*(−/−) mice at 48 h and by 50% at 72 h ($P < 0.05$; Fig. 2).

The mean numbers of BAL fluid PMNs, macrophages, and epithelial cells were not significantly different in *Nos2*(−/−) and *Nos2*(+/+) mice exposed to filtered air for 48 or 72 h (Table 2). Significant increases in the mean number of PMNs, macrophages, and epithelial cells were found in *Nos2*(−/−) and *Nos2*(+/+) mice after 48 and 72 h of exposure to O_3 ($P < 0.05$; Table 2). However, the mean cell numbers were not significantly different between *Nos2*(−/−) and *Nos2*(+/+) mice at either 48 or 72 h (Table 2).

Regulation of O_3-induced increase in Nos2 levels by Tlr4. Kleeberger et al. (23) demonstrated previously that basal levels of *Tlr4* mRNA in the lungs of HeJ and OuJ mice (which differ only at the *Tlr4* locus) were not significantly different from each other. Exposure to O_3 for 72 h caused *Tlr4* mRNA levels to decrease beyond detection in the lungs of O_3-resistant HeJ mice but increase in O_3-susceptible OuJ mice (23). To determine whether differential susceptibility to O_3-induced hyperpermeability in HeJ and OuJ mice is regulated through iNOS, we compared expression kinetics of *Nos2* and *Tlr4* in both strains. Mice were exposed to filtered air or O_3 for 1.5, 3, 6, 24, 48, and 72 h. The mean total BAL fluid protein concentration was not significantly different between the strains at any time after air exposure. No differences in BAL fluid protein between HeJ and OuJ mice were found after 1.5, 3, or 6 h of O_3 exposure. However, as previously demonstrated (23), BAL fluid protein concentration was significantly elevated in OuJ mice compared with that in HeJ mice after 24, 48 and 72 h of exposure to O_3 (Table 3).

Table 1. *Effect of L-NMMA on the no. of macrophages, PMNs, and epithelial cells recovered by BAL*

Cell Type	Treatment	No. of Cells, ×10³/ml BAL return	
		Air	Ozone
Macrophages	L-NMMA	24.3 ± 4.8	42.7 ± 6.3*
	Vehicle	25.7 ± 5.3	48.3 ± 6.2*
PMNs	L-NMMA	0.1 ± 0.1	3.6 ± 1.0*
	Vehicle	0.1 ± 0.1	5.4 ± 0.9*
Epithelial cells	L-NMMA	2.6 ± 0.2	4.0 ± 0.5*
	Vehicle	2.9 ± 0.3	3.9 ± 0.5*

Values are means ± SE; n = 4–6 animals/group. L-NMMA, N^{G}-monomethyl-L-arginine; PMNs, polymorphonuclear leukocytes; BAL, bronchoalveolar lavage; vehicle, Hanks' balanced salt solution. Cells were recovered by BAL after C57BL/6J mice were exposed for 72 h to 0.3 parts/million (ppm) ozone and filtered air. $^{*}P < 0.05$ compared with air.

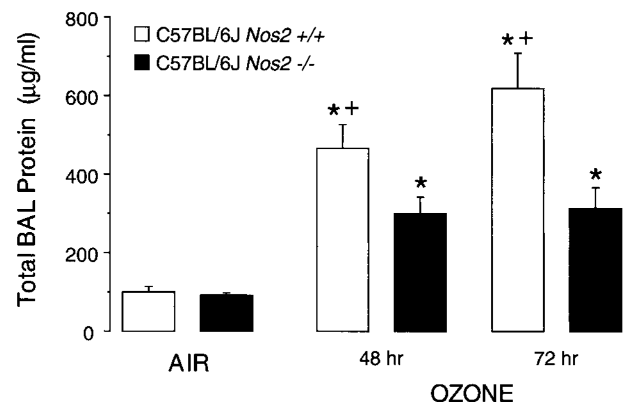

Fig. 2. Site-directed mutation of inducible nitric oxide synthase gene (*Nos2*) in C57BL/6J mice significantly attenuated the permeability response induced by 48 and 72 h of exposure to 0.3 ppm O_3 (n = 5–8/group). *Nos2*(+/+), *Nos2* wild type; *Nos2*(−/−), *Nos2* knockout. Air control mice were not significantly different from each other and were pooled. $^{*}P < 0.05$ vs. air. $^{+}P < 0.05$ vs. *Nos2*(−/−).

Table 2. *Effect of site-directed mutation of Nos2 on the no. of macrophages, PMNs, and epithelial cells recovered by BAL*

		No. of Cells, $\times 10^3$/ml BAL return			
		Air		Ozone	
Cell Type	Genotype	48 h	72 h	48 h	72 h
Macrophages	*Nos2*(+/+)	37.4 ± 2.9	39.4 ± 2.6	67.9 ± 4.4*	64.2 ± 9.6*
	Nos2(−/−)	36.3 ± 4.6	32.1 ± 6.8	82.7 ± 2.4*	52.7 ± 12.0*
PMNs	*Nos2*(+/+)	0.8 ± 0.4	0.8 ± 0.4	4.5 ± 0.4*	6.6 ± 1.0*
	Nos2(−/−)	0.9 ± 0.4	1.4 ± 0.4	5.7 ± 0.6*	4.5 ± 0.7*
Epithelial cells	*Nos2*(+/+)	2.7 ± 0.5	2.7 ± 0.4	5.4 ± 0.6*	6.8 ± 1.0*
	Nos2(−/−)	3.1 ± 0.6	2.7 ± 0.7	6.4 ± 1.2*	4.5 ± 1.0*

Values are means ± SE; n = 4–6 animals/group. *Nos2,* gene for inducible nitric oxide synthase; *Nos2*(+/+), *Nos2* wild-type [C57BL/6J-*Nos2*(+/+)]; *Nos2*(−/−), *Nos2* deficient [C57BL/6J-*Nos2*(−/−)]. Cells were recovered by BAL after mice were exposed to 0.3 ppm ozone and filtered air for indicated times. *P < 0.05 compared with time-matched air control mice.

In the lungs of HeJ and OuJ mice exposed to air, *Nos2* mRNA levels were not different between the strains (Fig. 3). However, *Nos2* mRNA levels differed markedly in the two strains after exposure to O_3. In the HeJ strain, mRNA levels were elevated slightly after 90 min of exposure to O_3 compared with those found after air exposure; mRNA levels decreased thereafter up to 24 h and remained depressed throughout the exposure. In OuJ mice, *Nos2* mRNA levels increased relative to the respective air control levels after 1.5 h of exposure to O_3. However, in contrast to the HeJ strain, *Nos2* mRNA did not change significantly throughout the exposure but remained elevated. β-Actin control levels were not different between strains or exposures (Fig. 3). The kinetics of *Tlr4* mRNA levels in HeJ and OuJ mice largely reflected those of *Nos2* mRNA. There were no differences between HeJ and OuJ mice after air exposure (Fig. 4). After 1.5 h of O_3 exposure, *Tlr4* mRNA levels decreased in HeJ mice and remained low throughout the 72-h exposure (Fig. 4). In contrast, *Tlr4* mRNA levels in OuJ mice remained the same as those in air-exposed control mice or increased during the O_3 exposure. β-Actin control levels were not different between the strains or exposures (Fig. 4).

Table 3. *Total BAL protein responses to 0.3 ppm ozone and filtered air in C3H/HeJ and C3H/HeOuJ mice*

Exposure Time, h	Exposure	Total Protein Concentration, μg/ml BAL return: C3H/HeJ	C3H/HeOuJ
1.5	Air	90 ± 9	105 ± 8
	Ozone	66 ± 6	100 ± 5
3	Air	94 ± 10	80 ± 11
	Ozone	83 ± 11	65 ± 6
6	Air	88 ± 8	106 ± 10
	Ozone	93 ± 11	128 ± 13
24	Air	126 ± 9	128 ± 12
	Ozone	282 ± 85*	558 ± 103*†
48	Air	100 ± 18	130 ± 18
	Ozone	331 ± 61*	634 ± 73*†
72	Air	118 ± 12	125 ± 11
	Ozone	327 ± 53*	612 ± 59*†

Values are means ± SE; n = 4–6 animals/group. *P < 0.05 compared with air. †P < 0.05 compared with C3H/HeJ mice.

DISCUSSION

In the present study, we demonstrated that NOS and, in particular, iNOS have an important role in O_3-induced lung hyperpermeability in B6 mice. This conclusion is supported by two experiments. First, treatment of B6 mice with a NOS inhibitor (L-NMMA) significantly decreased the change in mean BAL fluid protein induced by O_3 relative to that in vehicle-treated mice. Second, the change in mean BAL fluid protein induced by O_3 in *Nos2* knockout mice (*Nos2*-deficient mice) was significantly reduced compared

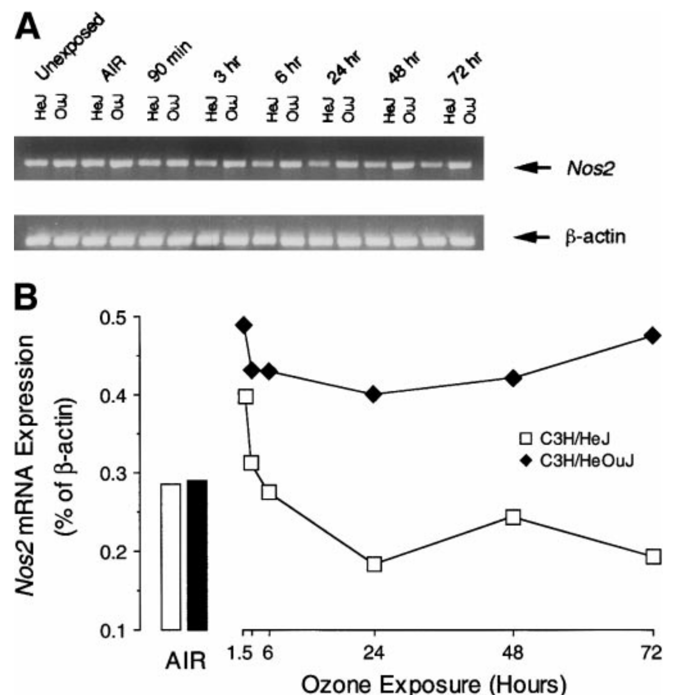

Fig. 3. *Nos2* mRNA is differentially expressed in C3H/HeJ mice compared with that in C3H/HeOuJ mice after exposure to 0.3 ppm O_3 for the indicated times. *A*: *Nos2* mRNA levels in lung tissue from C3H/HeJ (HeJ) and C3H/HeOuJ (OuJ) mice as detected by RT-PCR. Simultaneous analysis of β-actin gene expression was done as a positive control. Tissues from 4–6 animals/group were pooled for the analyses. *B*: quantitation of relative *Nos2* gene expression in C3H/HeJ and C3H/HeOuJ mice after air and O_3 exposure. Data are expressed after normalization to respective β-actin control.

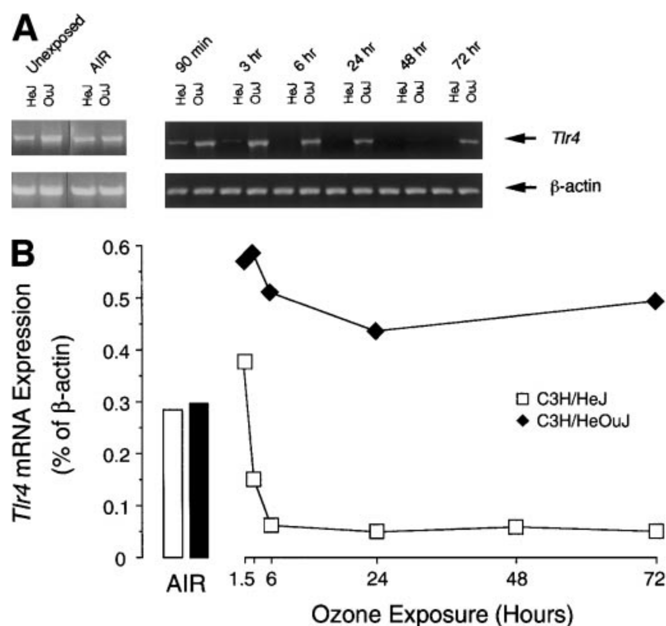

Fig. 4. Toll-like receptor 4 (*Tlr4*) mRNA is differentially expressed in C3H/HeJ compared with C3H/HeOuJ mice after exposure to 0.3 ppm O_3 for the indicated times. *A*: *Tlr4* mRNA levels in lung tissue from C3H/HeJ and C3H/HeOuJ mice as detected by RT-PCR. Pooled cDNA for OuJ mice at 48 h did not amplify; therefore, no band was visualized. Simultaneous analysis of β-actin gene expression was done as a positive control. Tissues from 4–6 animals/group were pooled for the analyses. *B*: quantitation of relative *Tlr4* gene expression in C3H/HeJ and C3H/HeOuJ mice after air and O_3 exposure. Data are expressed after normalization to respective β-actin control.

with that in wild-type B6 mice after 48 and 72 h of exposure. It is important to note that neither experiment accounted for all the O_3-induced permeability change. In *Nos2* knockout mice, ~50% of the hyperpermeability was inhibited relative to that in wild-type mice, whereas L-NMMA inhibited 35% of the O_3 effect relative to vehicle control mice. Additional contributors to O_3-induced hyperpermeability may include lipid mediators and cytokines. For example, platelet-activating factor has been shown to have a potent effect on airway permeability in the mouse, and a role for this mediator has been demonstrated in O_3-induced acute lung injury (33).

It is interesting that the L-NMMA treatment, which putatively blocks all NOS activity, was a less effective inhibitor of O_3-induced hyperpermeability than targeting of the iNOS gene (*Nos2*). This apparent discrepancy is likely due to differences in the specificity of the two treatments. Although the dosing regimen for L-NMMA in B6 mice approximated the effective doses published elsewhere (e.g., Ref. 32), the duration of those studies was relatively short (4–6 h). The present experiments were considerably longer (3 days), and although a boosting dose was administered during the exposure, it is conceivable that the effective lung tissue dose was not optimal during the entire experimental protocol. On the other hand, targeted disruption of *Nos2* effectively ablates all gene function and, therefore, eliminates the contribution of iNOS to injury or inflammatory processes. In any case, results of both experiments were consistent and supportive of a role for NOS in this model.

The effects of L-NMMA and *Nos2* knockout on lung permeability were apparently not the result of reduced infiltrating inflammatory cells. In both experiments, O_3-induced increases in lavageable PMNs and macrophages were not affected by inhibition of NOS. This suggests that NO is not involved in the chemotactic pathway that results in PMN and macrophage infiltration into the lower airways after subacute O_3 exposure. These observations are consistent with those of Li et al. (32), who found that L-NMMA similarly inhibited lung hyperpermeability induced by instilled endotoxin in rats and that endotoxin-induced infiltration of macrophages and PMNs was not affected by the NOS inhibitor. However, another study (18) found that the NOS inhibitors N^G-nitro-L-arginine methyl ester and aminoguanidine reduced airway inflammation in guinea pigs exposed to 3 ppm O_3 for 2 h. The treatment effect was not observed until 5 h after exposure, and later postexposure time points were not reported. The discrepancy between this study and others (32) may be due to differences in species and exposure. Airway inflammation induced in mice by acute and subacute O_3 exposures have different genetic mechanisms (22), and it is possible that, in contrast to the subacute model presented here, the inflammatory cell response to acute O_3 exposure has a NO-dependent component.

Our experiments do not exclude the possibility that macrophages and PMNs are important contributors to the permeability changes induced by O_3. Both cell types have the capacity to produce NO when stimulated (8, 34) and thus may be important sources of NO in the lung response to subacute O_3 exposure. Furthermore, it is likely that both treatments (inhibition of NOS and targeted disruption of *Nos2*) inhibited NO production in macrophages and PMNs. Experiments designed to deplete PMNs (e.g., Ref. 21) or inhibit macrophage function (e.g., Ref. 39) are necessary to address the specific role of these cells in NO-mediated changes in lung permeability. Experiments designed to inhibit cell function must be interpreted carefully, however, because PMNs and macrophages have the capacity to release many de novo synthesized and stored products that could contribute to hyperpermeability [e.g., platelet-activating factor (33)].

Collectively, the present studies are consistent with a role for NO in O_3-induced hyperpermeability in the mouse. However, the mechanisms through which iNOS is regulated (and NO production increased) during O_3 exposure are not understood. We hypothesize that the involvement of iNOS in O_3-induced hyperpermeability is mediated through *Tlr4*. The role of *Tlr4* as a critical component in the innate immune responses has been well described (26, 27). The involvement of NO in the innate immune effector pathways has also been defined (see Ref. 3). Furthermore, Ohashi et al. (36) have shown in bone marrow-derived macrophages from HeJ and endotoxin-responsive C3H/HeN mice that NO formation is dependent on a functional *Tlr4*. Previous

work from our laboratory (23) and the present study also indicate that *Tlr4* is an important regulator of lung hyperpermeability induced by O_3 exposure. Stimulation of *Tlr4* initiates an intracellular signaling pathway that utilizes a series of adapter proteins and serine/threonine kinases that link to the protein kinase nuclear factor (NF)-κB-inducing kinase (26, 27). NF-κB subsequently induces key effector genes, including those for proinflammatory cytokines (15, 53). There is accumulating evidence that *Nos2* regulation is also mediated, in part, through activation of NF-κB (10, 19, 34). Because of the commonality between mechanisms of endotoxin- and O_3-induced lung hyperpermeability, we investigated whether the involvement of iNOS in O_3-induced hyperpermeability is mediated through *Tlr4*. Differences in *Nos2* gene expression between O_3-exposed HeJ and OuJ mice were consistent with this hypothesis. *Nos2* expression was markedly decreased in HeJ mice after 6 h of exposure to O_3, whereas *Nos2* expression in OuJ mice was elevated during exposure. To further examine the relationship between *Tlr4* and *Nos2*, the expression kinetics of both genes were compared in HeJ and OuJ mice. Interestingly, the kinetics of *Tlr4* and *Nos2* expression were correlated in both strains after exposure to O_3. Although *Tlr4* mRNA levels were expressed constitutively and similarly in both strains, levels were downregulated in HeJ lung homogenates as early as 90 min after exposure to O_3 and were maintained throughout the 72-h exposure. The pattern of O_3-induced change in *Tlr4* gene expression therefore resembled that of *Nos2* in HeJ mice. In contrast to HeJ mice, *Tlr4* mRNA levels in the OuJ strain increased early and remained elevated for up to 72 h. The correlative patterns of gene expression in the two strains therefore support a role for *Tlr4* in the regulation of *Nos2* during O_3 exposure in the mouse.

The mechanisms through which NO causes a change in epithelial permeability in this model have not been determined. NO generated by NOS will react with superoxide anions to produce the highly oxidative molecule peroxynitrite, which may further lead to the formation of nitrotyrosine. Peroxynitrite and nitrotyrosine are highly cytotoxic and have been associated with acute lung injury (16, 25) and microvascular hyperpermeability (46). Thus it is conceivable that NO-mediated hyperpermeability caused by O_3 exposure may be induced by secondary production of cytotoxic molecular species. It is also possible that hyperpermeability induced by O_3 could be due, in part, to an uneven distribution of blood flow. Acute exposures to high concentrations of O_3 cause increased bronchial circulation in sheep, which may contribute to the changes in lung permeability observed in this model (11, 44). Because NO has potent vasodilatory properties, O_3-induced release of this gas into the microenvironment may alter blood flow and increase vessel permeability.

Another possibility is that NO effects are mediated through the regulation of downstream effector genes. NO has been demonstrated to modulate the transcription factor NF-κB, which, in turn, regulates cytokines that are critical to the development and maintenance of pulmonary inflammation (2). The effect of NO on NF-κB is primarily inhibitory. For example, de la Torre et al. (7) demonstrated that lipopolysaccharide-mediated NO synthesis is associated with S-nitrosylation of NF-κB p50 that inhibits NF-κB-dependent gene transcription in ANA-1 murine macrophages. However, NO may also activate NF-κB in some cell types (see Ref. 35). Clearly, further work is necessary to understand the mechanisms through which NO may be mediating the hyperpermeability effect of O_3, and work is ongoing in our laboratory to address this question.

Activation of TLR4 receptors by O_3 is not clearly understood. It may be speculated that lipid ozonation products (LOPs) and other products of oxidant interaction with molecules in the epithelial lining fluid and cell membranes may stimulate TLR4 receptors. Kafoury et al. (20) have suggested that a cascade of events initiated by LOPs leads to inflammation in the lung, but it remains to be demonstrated whether LOPs initiate these processes via TLR4 receptors.

In summary, the inhibitory effects of L-NMMA on O_3-induced BAL fluid protein and differences in BAL fluid protein between *Nos2*(+/+) and *Nos2*(−/−) mice after O_3 exposure are consistent with a role for iNOS in the development of oxidant-induced lung hyperpermeability. Furthermore, differential expression of *Nos2* in HeJ and OuJ mice during O_3 exposure suggests that *Tlr4* has an important role in the modulation of *Nos2*.

We thank Kiana Brunson for excellent technical assistance.

This study was supported by National Institute of Environmental Health Sciences Grants ES-03819 and ES-09606; National Heart, Lung, and Blood Institute Grants R01-HL-57142 and R29-HL-58122; and Environmental Protection Agency Grant EPA R-826724.

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