increased production of nitrotyrosine in lung tissue of rats with radiation-induced acute lung injury

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Departments of 1Physiology and 2Internal Medicine, Tokai University School of Medicine, Isehara, Kanagawa 259-11; and 3Electro-Chemical and Cancer Institute, Chofu, Tokyo 182-0022, Japan

Tsui, Chizuko, Sumie Shioya, Yuki Hirota, Naoto Fukuyama, Daisaku Kurita, Toshimori Tanigaki, Yasuyo Ohta, and Hiroe Nakazawa. Increased production of nitrotyrosine in lung tissue of rats with radiation-induced acute lung injury. Am J Physiol Lung Cell Mol Physiol 278: L719–L725, 2000.—The purposes of this study were 1) to identify the nitric oxide (NO) synthase (NOS) isoform responsible for NO-mediated radiation-induced lung injury, 2) to examine the formation of nitrotyrosine, and 3) to see whether nitrotyrosine formation is reduced by an inducible NOS (iNOS) inhibitor, aminoguanidine. We conclude that iNOS induction is a major factor in radiation-induced lung injury and that nitrotyrosine formation may participate in the NO-induced pathogenesis.

Materials and Methods

Animals

Specific pathogen-free male Wistar rats (n = 44) aged 9–10 wk (SLC, Shizuoka, J apan) were used. All rats were kept under specific pathogen-free conditions until used. All experiments complied with the guidelines for animal experiments of the School of Medicine, Tokai University (Kanagawa, J apan).

Experimental Protocol

The experiments were performed 2 wk after irradiation because acute lung damage peaked at week 2 in previous studies by Kawana et al. (9) and Shioya et al. (19). The early damage appears to play a key role in initiating the inflammatory cascade that leads to the development of radiation pneumonitis and fibrosis.

The rats were anesthetized with 50 mg/kg of pentobarbital sodium intraperitoneally, and the left hemithorax was irradiated with an absolute dose of 20 Gy in one fraction, delivered by a 60Co teletherapy unit (Theratron, Ottawa, Canada). The right hemithorax and other organs were shielded during irradiation. The rats were killed with an overdose of pentobar-
bitol sodium, and the left lungs were isolated for NMR measurement, histological examination, and measurement of NOS mRNA and tissue nitrotyrosine. For histological examination, the lungs were fixed with Formalin-saline, and for measurement of NOS mRNA and nitrotyrosine formation, the lungs were frozen with liquid nitrogen and stored at -80°C until used. For bronchoalveolar lavage (BAL), two different groups of rats were prepared. One group of rats was prepared to obtain inflammatory cells in BAL fluid (BALF) and lung tissue for the measurement of superoxide (O$_2^-$) production. The other group was prepared to obtain BALF for measurement of the concentrations of nitrite and nitrate (NO$_2^-$/NO$_3^-$) and protein and the activity of lactate dehydrogenase (LDH). BAL was performed only in the irradiated left lung immediately after death through a plastic tube inserted in the left main bronchus while the right main bronchus was ligated.

Experimental Groups

The rats were divided into the following four groups: 1) control group (C group), 2) irradiated group (R group), 3) radiation plus aminoguanidine (AG) group (R+AG group), and 4) aminoguanidine group (AG group). In the R+AG group, the rats were treated with aminoguanidine 2 h before irradiation followed by a daily subcutaneous injection of 50 mg/kg and oral administration of 2 g/l in the drinking water. In the AG group, AG was provided on the same schedule as in the R+AG group.

Measurement of NOS mRNA

The frozen lung tissue was homogenized in ISOGEN (Nippon Gene, Tokyo, Japan), and RNA was extracted. The expression of mRNA for iNOS and endothelial NOS (eNOS) was measured with the RT-PCR method as previously described (14, 20). Briefly, equal amounts of RNA were reverse transcribed into cDNA. The RT products were amplified with primers for both iNOS and eNOS designed from rat gene sequences (14, 20), and gyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a standard. After amplification, each PCR mixture was electrophoresed through 1% agarose gel, which was stained with ethidium bromide. Each gel was photographed under ultraviolet (UV) light with the same exposure and development time. The bands of the positive film were scanned, and the density of each PCR product was evaluated with National Institutes of Health (NIH) Image software. The ratio of NOS gene product to GAPDH gene product was used for semiquantification.

Measurement of NO$_2^-$/NO$_3^-$ and Protein Concentrations and LDH Activity in BALF

The left lung was lavaged with heparinized (20 U/ml) saline (2 ml) three times, and an average of 1.4 ml of BALF was obtained. NO$_2^-$/NO$_3^-$, stable end products of NO, was assayed with the Griess reaction (11) after the cells were removed by centrifugation and a molecular-weight cutoff filter (mol wt 5,000; 15,000 rpm for 40 min) was used to remove proteins that disturb the measurement. Total NO$_2^-$/NO$_3^-$ was measured with an HPLC system (JASCO, Tokyo, Japan) equipped with a cadmium column to reduce nitrate to nitrite and a UV-visible absorbance detector (550 nm; JASCO).

The protein concentration and LDH activity in BALF were measured as indexes of lung damage with a modification of the Lowry method and an enzyme assay kit (Promega, Madison, MI), respectively.

Fig. 1. Expression of inducible nitric oxide synthase (iNOS; A) and endothelial NOS (eNOS; B) mRNAs in rat lung. Top: ethidium bromide-stained agarose gels of amplified iNOS (826 bp) or eNOS (819 bp) and housekeeping gyceraldehyde-3-phosphate dehydrogenase (GAPDH; 268 bp) PCR products. Bottom: relative semiquantitative assessment of PCR products with National Institutes of Health Image software. C, control group (n = 4 rats); R, irradiated group (n = 4 rats); R+AG, aminoguanidine-treated irradiated group (n = 3 rats). Values are means ± SD expressed as ratio of NOS to GAPDH mRNA (NOS/GAPDH).

Fig. 2. Concentration of nitrite and nitrate (NO$_2^-$/NO$_3^-$) in bronchoalveolar lavage fluid (BALF) as measured by Griess assay method. Values are means ± SD; n = 4 rats/group.
Measurement of NMR Transverse Relaxation Time in Lung Tissue

NMR relaxation analysis provides information about the molecular damages in the tissue through interactions between the protons of water and the protons of macromolecules. The spin-spin relaxation time (T2) is a sensitive measure of detecting tissue damage even when there was no evidence of damage on histological examination in the early stage of radiation lung injury (19). Immediately after death, a sample 2 mm in diameter and 8 mm in length was excised from the passively collapsed left lung. Each sample was placed in a 90-MHz Fourier-transform NMR spectrometer (FX90A, JEO, Tokyo, Japan), and NMR T2 was measured by the Carr-Purcell-Meiboom-Gill method. The spin-echo signals were Fourier transformed, and the T2 decay curves for water peak were determined. The T2 decay curves of peripheral lung tissue have been shown to be multiexponential and can be fitted with two components. Values of the two T2 components, fast (T2f) and slow (T2s), which reflect changes in the intracellular and extracellular water, respectively, were determined by an iterative least squares curve fitting (19).

Biochemical Detection of Nitrotyrosine Formation in the Lung

The frozen lung was homogenized with Milli-Q water and hydrolyzed as previously reported (6). Briefly, homogenates were hydrolyzed in 0.1% phenol containing 6 N HCl at 110°C in a vessel rack (Jasco) for 24 h. Separation of tyrosine and nitrotyrosine was achieved by HPLC on a Nucleosil 5-µm C-18 reverse-phase column (15 cm x 4.6 mm) with a guard column (Jasco). The column was eluted with 50 mmol/l of KH2PO4-H3PO4 (pH 3.01) containing 10% methanol at a flow rate of 1 ml/min through an isocratic pump, and the peaks were measured with an UV detector set at 274 nm. The level of nitrotyrosine is expressed as the percentage of nitrotyrosine to total tyrosine.

Immunohistochemical Detection of Nitrotyrosine

A paraffin-embedded sample was sliced and treated with 0.1% trypsin in Tris-HCl buffer. Endogenous peroxidase was blocked with hydrogen peroxide in methanol. Nitrotyrosine was stained with nitrotyrosine antibody (2). Antibody binding was then visualized by a diaminobenzidine-peroxidase reaction with a peroxidase-labeled second antibody (mouse and rabbit cocktail). A negative control was prepared in each group with the same protocol except for the primary antibody to exclude nonspecific staining. The specimens were counterstained with methyl green.

Fig. 3. Protein concentration (A) and lactate dehydrogenase (LDH) activity (B) in BALF. Protein concentration was measured by modified Lowry method, and LDH activity was measured with enzyme assay kit. AG, aminoguanidine control group. Values are means ± SD; n = 4 rats/group.

Fig. 4. Changes in NMR spin-spin relaxation time (T2) of lung tissue. A: fast T2 component (T2f). B: slow T2 component (T2s). A sample of peripheral lung tissue 2 mm in diameter and 8 mm in length was excised from passively collapsed left lung. NMR T2 was measured by Carr-Purcell-Meiboom-Gill method with a 90-Hz Fourier-transform NMR spectrometer. Values are means ± SD; n = 4 rats/group.
Measurement of O$_2^\cdot$ Production by BALF Cells and Tissue Cells

For the examination of BALF cells, we removed blood by perfusing Krebs-Ringer phosphate buffer through the pulmonary artery at 37°C before the BAL procedure. The left lung was lavaged with 2-ml aliquots of heparinized saline 12 times (a total of 24 ml for each lung). An average of 23.4 ml of BALF was recovered. BALF cells were obtained by centrifugation at 480 g at 4°C for 10 min and were suspended in phosphate-buffered saline (PBS).

Because inflammatory cells that invade the lung tissue (lung cells) cannot be collected by the BAL procedure, tissue cells were obtained from the left lung after the BAL procedure as previously described (28). Briefly, the tissue was minced and incubated in Hanks’ balanced salt solution with collagenase and DNase at 37°C for 90 min, then filtered through a cloth (32-µm pore diameter) into RPMI 1640 medium containing 10% fetal calf serum. The adherent cells, which include AMs, polymorphonuclear neutrophils (PMNs), and interstitial macrophages were resuspended in PBS until used.

The total number of BALF and lung cells was counted with a hemocytometer. Composition of the cells was performed on cytospun preparations stained with Diff-Quik hematoxylin and eosin (Cytospin, Shandon Instruments, Cheshire, UK).

O$_2^\cdot$ production was measured with the chemiluminescence method with an O$_2^\cdot$-specific chemiluminescent probe, 2-methyl-6-(p-methoxyphenyl)-3,7-dihydropyrazin-3-one (Tokyo Kasei, Tokyo, Japan) (12) in the presence of 50 ng of phorbol 12-myristate 13-acetate as previously described (23). To terminate O$_2^\cdot$ production, 50 U of superoxide dismutase were added. The amount of O$_2^\cdot$ production is expressed as counts per second per cell x total cell count.

Statistical Analysis

Data are expressed as means ± SD. To compare more than three groups, Tukey’s multiple comparison test was used (29). A probability value of 0.05 or less was considered significant.

RESULTS

NOS Induction and NO$_2^-$/$\text{NO}_3^-$ Generation

Figure 1 shows the expression of mRNA for iNOS (A) and eNOS (B) in lung tissues as measured by the RT-PCR method. Both NOS mRNAs were detected in all groups, but expression was increased in the R and R+AG groups. The values of the iNOS-to-GAPDH mRNA ratio were 0.033 ± 0.040, 0.189 ± 0.031, and 0.152 ± 0.013 and those of the eNOS-to-GAPDH mRNA ratio were 0.061 ± 0.065, 0.346 ± 0.128, and 0.421 ± 0.013 in the C, R, and R+AG groups, respectively.

Figure 2 shows the concentration of NO$_2^-$/$\text{NO}_3^-$ in BALF. Although an increase in vascular permeability in the R group may influence the results, NO$_2^-$/$\text{NO}_3^-$ was significantly increased in the R group, but the increase was attenuated in the R+AG group. There was no

Fig. 5. Nitrotyrosine formation in lung. After lung tissue was hydrolyzed, nitrotyrosine and tyrosine were separated by HPLC and measured with ultraviolet detector. nd, Not detected. Values are means ± SD expressed as percentage of nitrotyrosine to total tyrosine (nitrotyrosine/tyrosine); n = 4 rats/group.

Fig. 6. Immunostaining with antiserum to nitrotyrosine in lung tissue. Nitrotyrosine formation is shown as a brown color with peroxidase-labeled second antibody by using diaminobenzidine-peroxidase reaction. NC, negative control group in R group. In R group, alveolar epithelium and macrophages were positively stained for nitrotyrosine (arrowheads). In R+AG group, macrophages are stained. NC group was prepared with the same protocol except for primary antibody.
significant difference in values between the C and R+AG groups.

Lung Injury

We measured the protein concentration and LDH activity in BALF as indexes of lung injury (Fig. 3, A and B, respectively). Protein concentration and LDH activity were increased in the R group, but these increases were completely prevented by AG treatment. Changes in the NMR relaxation times T2f and T2s of the lung are shown in Fig. 4. The T2f and T2s increased in the R group, and AG treatment attenuated the increase in T2f and T2s, although the T2f in the R+AG group increased compared with that in the C group, suggesting mild interstitial damage. In the AG control group, there was no significant difference from the control value in protein concentration, LDH activity, or NMR T2.

Nitrotyrosine Formation

Nitrotyrosine was not detected in the C group, but it increased in the R group and the increase was attenuated by AG treatment (Fig. 5).

Immunohistochemical Detection of Nitrotyrosine

Nitrotyrosine staining was not observed in the C group but was present in the R group (Fig. 6). Nitrotyrosine-positive regions tended to coincide with sites of lung tissue injury, being located in the vicinity of airways, alveolar epithelia, and AMs. In the R+AG group, nitrotyrosine staining was not as marked as that in the R group.

Cellular Composition of Cells in BALF and in Lung Tissue

BALF cells. Figure 7 shows the total number and composition of BALF cells. The significant decrease in the number of BALF cells in the R and R+AG groups probably reflects the direct fatal effect of irradiation on the inflammatory cells that reside in lung tissue. Also, it may reflect a methodological limitation of the BAL procedure; i.e., firmly adhering cells cannot be lavaged. However, it is clear that the PMN fraction increases and monocytes emerge in the R and R+AG groups. O2 production showed no significant difference among groups.

Lung cells. The number of lung cells obtained was comparable in the C, R, and R+AG groups (10.39+4.77, 15.08+7.04, and 10.87+1.42, respectively; Fig. 8). AMs were decreased and PMNs were increased in the R group. The pattern of cell composition in the R+AG group was intermediate between those of the control and R groups. O2 production by total cells was significantly greater in the R and R+AG groups than in the C group (Fig. 9). However, there was no significant difference in O2 production between the R and R+AG groups.

DISCUSSION

In this study, we observed an increased expression of both eNOS and iNOS mRNAs together with an in-
increased concentration of NO$_2$/NO$_3$ in BALF from irradiated lung tissue after 2 wk. Treatment with an iNOS inhibitor attenuated the injury without affecting the expression of eNOS and iNOS. Although the increase in eNOS is a novel finding, an increase of iNOS in the irradiated lung was already reported by Nozaki et al. (13), who showed that the inhibition of NO formation by N-nitro-l-arginine methyl ester, a nonspecific NOS inhibitor, attenuated the injury. In this study, we were able to show that a relatively iNOS-specific inhibitor, AG, attenuated all indexes of the injury, indicating that iNOS, not eNOS, is the major source of NO production and is primarily responsible for inducing the injury. Although the role of eNOS in radiation-induced lung injury remains to be determined, it may represent regenerating epithelial cells because a marked increase in eNOS occurs in denuded aorta or in the repair stage of inflammation (16, 18).

The nitrotyrosine-to-total tyrosine ratio was increased in the irradiated lung. Furthermore, nitrotyrosine staining was prominent in the areas where the destruction was severe. The nitrotyrosine-positive cells were identified as AMs and epithelial cells of the alveolus and airways. These cell types are known to express iNOS protein and mRNA (13), indicating that they may be major sources of NO production. Although nitrotyrosine formation was decreased by AG treatment in terms of both biochemical and immunohistochemical criteria, the degree of decrease was less compared with that in the injury because almost complete protection against injury except the change in MNR T$_2$ was observed. This suggests that tyrosine nitration is not the only mechanism causing injury. Another plausible mediator of the injury is O$_2^-$, which has been shown to play an important role in ischemia-reperfusion injury and hypoxic or hyperoxic lung injury (8, 21, 22). The generation of O$_2^-$ was increased in lung cells in the irradiated group compared with that in the C group. However, increased generation was also observed in the AG-treated group in which the injury was markedly attenuated, indicating a limited direct role of O$_2^-$ in causing the injury.


Chemiluminescence probe with Cypridina luciferin analog. 2-methyl-6-phenyl-3,7-dihydronaphazin-1,2-dialpyrazin-3-one, for estimating the ability of human granulocytes to generate O$_2^-$. Anal Biochem 159: 363–369, 1986.


