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INCREASED NITRIC OXIDE BIOSYNTHESIS IN LEUKOTOXIN, 9, 10-EPOXY-12-OCTADECENOATE INJURED LUNG

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Summary: We measured effluent nitric oxide levels using a chemiluminescence method from leukotoxin (Lx, a linoleate epoxide) injured isolated rat lungs perfused with physiological salt solution. Nitric oxide production from Lx-injured lung promptly increased and lasted for 20 min. Pretreatment with N^G-monomethyl-L-arginine (LNMMA) significantly suppressed Lx-induced production of nitric oxide. Effluent from control lungs showed trace levels of nitric oxide. The wet to dry lung weight (WLW/DLW) after termination of the experiments was significantly elevated in Lx-treated lungs compared with that of LNMMA pretreated lungs or control lungs. There was a correlation between nitric oxide levels (at 10 min) and lung edema (WLW/DLW). Thus, nitric oxide plays a role in the pathogenesis of Lx-induced lung injury.

Leukotoxin (Lx), a linoleate epoxide which is synthesized by neutrophils (1), was found to be increased in bronchoalveolar lavage fluid in patients with adult respiratory distress syndrome (ARDS) (2) and to cause ARDS-like lung injury when injected into animals (2). The precise injurious mechanism is still unclear, though Ozawa et al. proposed cell mitochondrial damage (2). Recently, we found that a smaller dose (which dose per se does not evoke lung injury) of Lx causes endothelium dependent pulmonary

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ABBREVIATIONS: Lx, leukotoxin; ARDS, adult respiratory distress syndrome; LNMMA, N^G-monomethyl-L-arginine; WLW/DLW, wet to dry lung weight.

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arterial ring relaxation and vasodilation in isolated perfused rat lungs (IPRL) (3). The Lx-induced edematous lung injury was inhibited in the presence of oxyhemoglobin, a nitric oxide trap or N^G- monomethyl-L-arginine (LNMMA) but not N^G- monomethyl-D-arginine (DNMMA) in IPRL (4).

We wondered whether nitric oxide is released during Lx-induced lung injury. We measured effluent nitric oxide levels by chemiluminescence and wet-to-dry lung weight ratio (WLW/DLW) as a marker of lung injury in Lx-treated IPRL.

MATERIALS AND METHODS

Materials. Lx was chemically synthesized from linoleate and purified by high pressure liquid chromatography as reported previously(1, 2, 3, 4). LNMMA was purchased from Calbiochem (La Jolla, CA) and Ficoll, meclofenamate and Earles balanced salt solution from Sigma (St Louis. MO). Sodium nitrite and ascorbate were purchased from Dojin (Kumamoto, Japan).

Isolated perfused rat lungs. Male Sprague-Dawley rats (280-350 g body wt.) were purchased from Japan Clea Inc. (Japan) and given food and water ad libitum. Lungs were isolated for ex vivo perfusion as described previously (5). Briefly, the lungs were ventilated through a tracheal cannula with a small-animal respirator (Shinano Seisakusho, Model SN-480-7, Tokyo) at 55 breaths/min with 7 cmH_2O maximal inspiratory pressure and 3 cmH_2O positive endoexpiratory pressure with a gas mixture containing 21 % O_2-5 % CO_2-74 % N_2. Following a median sternotomy and the intravenous injection of 100 IU heparin into the right ventricle, the pulmonary artery and left ventricle were cannulated. The lungs were perfused at constant flow(0.03 ml/g rat wt. /min) using a Masterflex pump PA-71A (Cole Parmer Instrument, Chicago, IL.) from a reservoir which contained 120 ml EBSS buffer, 4 g% Ficoll and 10 μM meclofenamate. The initial 50 ml of lung perfusate were discarded to remove blood components. The EBSS contained (in mM): NaCl 116.34, KCl 5.36, $MgSO_4$ 0.83, $NaHCO_3$ 19.04, $CaCl_2$ $2H_2O$ 1.80, $NaPO_4$ (dibasic) 0.40, glucose 5.50, Phenol redNa 0.03. Mean pulmonary arterial pressure (Ppa) was measured via a pressure transducer (Spectramed Co. P23XL) and weight gain by the lung (WLW) was measured via a force displacement transducer (EF601G Nihon koden, Japan). The lungs were allowed to equilibrated for 20 min to confirm the stability of Ppa and WLW. Then, Lx which was dissolved in ethyl alcohol (100 %, 20 μl) and mixed with EBSS buffer (40 μl) was injected into pulmonary cannula. As a control vehicle 20 μl ethyl alcohol + 40 μl EBSS buffer was injected in 5 perfused lungs. To demonstrate convincingly that the signal detected by chemiluminescence was due to nitric oxide, LNMMA 400 μM was added to the reservoir 8 min before Lx addition in 5 lungs. At the 20 min after Lx addition perfusion was terminated, each lung was removed for the measurement of WLW and dried in an oven at 56 °C for three weeks.

Chemiluminescence assay. Nitric oxide was measured using an NO chemiluminescence analyzer (FES-450 NO analyzer, Scholartec. Osaka). 3 ml effluent for NO analysis was serially collected into vials which were throughly rinsed with distilled water and then rinsed with ascorbate before, 5 min-, 10 min-, 15min-, and 20 min-after Lx addition. Ascorbate 6 ml of 3.13 M was added to the vials to reduce nitrite (NO_2-) (that is transformed by the interaction with O_2 and nitric oxide) present in the samples to nitric oxide and then vibrated for mixing. Vials were bubbled with N_2 (0.4 L/min) to

strip nitric oxide into head space. The head space gas was continuously drown into the analyzer and mixed with O3 generated by ozone generator (1 L/min). With use of an integration time of 100 msec, the light emission was detected by a cooled (at -4 °C)Hamamatsu red-sensitive photomultiplier tube (R 1477HA). The NO analyzer was interfaced with a computer (NEC 9801, Tokyo). Because EBSS-ficoll solution itself produced a signal (7.8 ± 0.6 pM, n=5), suggesting the presence of nitric oxide, nitrite, nitrate or nitrosothiols in the perfusate, this background signal was subtracted from signals obtained from the lung effluent. A standard curve, produced by sodium nitrite (1pM, 5 pM, 10 pM, 50 pM, 80 pM and 100 pM) was obtained (n=3 each) at the time of each perfusate sample measurement. The relationship between the dose of sodium nitrite and the signal (Fig. 1) was linear but at higher concentrations (50 pM) nonlinear; the sample values of our experiments were within the linear portion of the standard curve.

Statistics. Data are presented as mean ± SE. Differences between groups are tested by one-way repeated analysis of variance, using Scheffe's test for multiple comparison. Differences are considered significant when $P<0.05$.

RESULTS

Nitric oxide detection in effluent from IPRL. Sequential changes of nitric oxide levels in the effluent from lungs are shown (Fig. 2). Lx treated lungs produced a prompt increase in effluent nitric oxide which lasted for 20 min. The peak level was reached at 10 min after Lx addition (47.2 ± 4.1 pM). By contrast, LNMMA pretreated lungs caused no elevation of nitric oxide in the effluent with transient suppression at 5 min. Control (vehicle-treated) lungs showed a stable production of nitric oxide during the course.

The relationship between nitric oxide production and WLW/DLW. Since peak levels of effluent nitric oxide were reached at 10 min after Lx addition, we looked at the correlation between the nitric oxide levels at 10 min and the WLW/DLW (Fig. 3). There was a significant correlation between these two variables ($r=0.545$, $p<0.02$, $n=15$). The WLW/DLW of Lx treated lungs (12.942 ± 0.68, n=5) was significantly greater ($p<0.05$) than

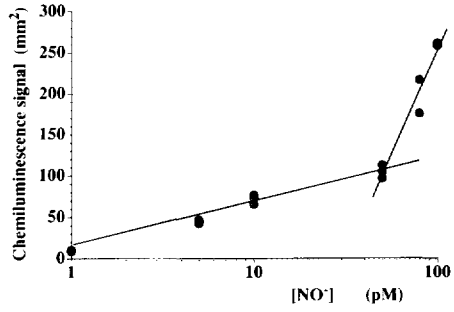

Figure 1. Standard curve obtained with sodium nitrite (1 pM, 5pM, 10 pM, 50 pM, 80 pM, and 100 pM, n=3 each).
Standard curve is linear at lower concentration but nonlinear at higher concentration (>50 pM).

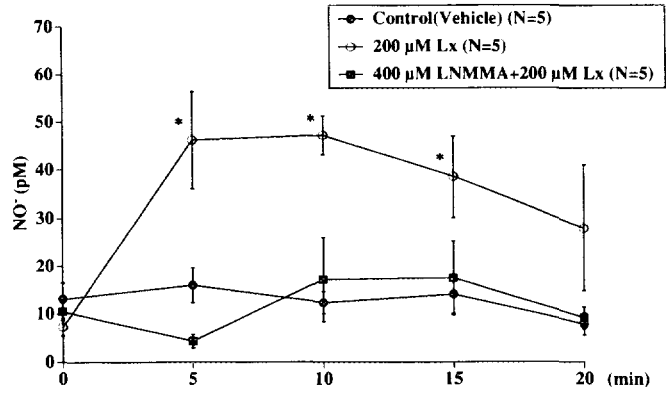

Figure 2. Sequential nitric oxide concentration in effluent during 20 min perfusion. Values are mean ± SE of 5 observations. Peak nitric oxide level was reached at 10 min after Lx-addition. * $P < 0.05$.

that of vehicle control (5.70 ± 0.1, n=5). Pretreatment of lungs with LNMMA significantly suppressed the Lx-induced increase in WLW/DLW; 8.99 ± 0.26, n=5. There was no significant difference in the baseline perfusion pressure or baseline wet lung weight among the three groups.

DISCUSSION

Our experimental results show that Lx increased the effluent nitric oxide levels and that WLW/DLW concordantly increased with the level of effluent nitric oxide. In addition, LNMMA suppressed both the Lx-induced increase in WLW/DLW and the Lx-induced increase in effluent nitric oxide. These results provide direct evidence of nitric oxide release from Lx-injured lungs, supporting our hypothesis that nitric oxide or a nitric oxide-related substance participate in Lx-induced lung injury.

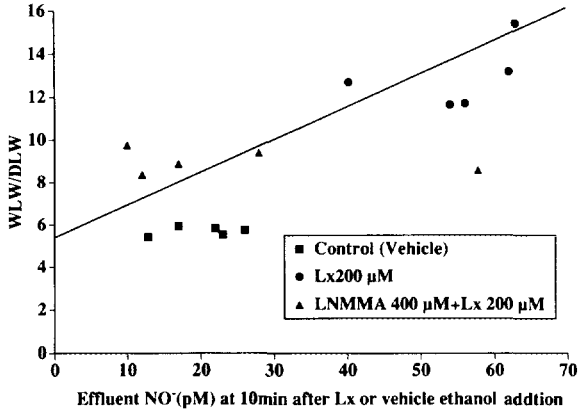

Figure 3. The relationship between effluent nitric oxide level at 10 min and WLW/DLW. $Y = 0.118 X + 6.186$, $r2 = 0.545$, $P < 0.02$.

Hitherto, nitric oxide detection has been based on gas chromatography-mass spectrometry(6), electron paramagnetic resonance spectroscopy (6, 7), chemiluminescence (6, 7), colorimetry (6, 8), high performance liquid chromatography (9), and capillary electrophoresis(10). Among these methods, chemiluminescence is well known to be sensitive to detect nitric oxide in biological samples. To our kowledge, however, the application of the chemiluminescence method to detect nitric oxide in effluents from isolated perfused lungs has been scarce. Isaacson et al. (11) reported that measurable amounts of effluent nitric oxide from chronically hypoxic but not normoxic perfused lungs. Our modified chemiluminescence method seems to be highly sensitive since basal release of nitric oxide from normoxic perfused lungs was detectable. It is possible that our values of nitric oxide in our study are a low estimate since nitric oxide may remain sequestered in subcellular components of the lung tissue (12) and rapid oxidation to nitrate during the passage of effluent from the left ventricle to the sampling site (15 cm long) may occur. However, nitrite, another nitric oxide oxidative product is completely reduced to nitric oxide by the sample pretreatment with saturated ascorbate. The fact that Lx causes an immediate release of nitric oxide from the lung suggests that Lx stimulates the constitutive endothelial nitric oxide synthase.

In conclusion, nitric oxide or a nitric oxide related substance is involved in the pathogenesis of Lx-induced lung injury.

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