Oxidative stress augments pulmonary hypertension in chronically hypoxic mice overexpressing the oxidized LDL receptor

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Ogura S, Shimosawa T, Mu S, Sonobe T, Kawakami-Mori F, Wang H, Uetake Y, Yoshida K, Yatomi Y, Shirai M, Fujita T. Oxidative stress augments pulmonary hypertension in chronically hypoxic mice overexpressing the oxidized LDL receptor. Am J Physiol Heart Circ Physiol 305: H155–H162, 2013. First published May 17, 2013; doi:10.1152/ajpheart.00169.2012.—Chronic hypoxia is one of the main causes of pulmonary hypertension (PH) associated with ROS production. Lectin-like oxidized low-density lipoprotein receptor (LOX)-1 is known to be an endothelial receptor of oxidized low-density lipoprotein, which is assumed to play a role in the initiation of ROS generation. We investigated the role of LOX-1 and ROS generation in PH and vascular remodeling in LOX-1 transgenic (TG) mice. We maintained 8- to 10 wk-old male LOX-1 TG mice and wild-type (WT) mice in normoxia (room air) or hypoxia (10% O2 chambers) for 3 wk. Right ventricular (RV) systolic pressure (RVSP) was comparable between the two groups under normoxic conditions; however, chronic hypoxia significantly increased RVSP and RV hypertrophy in LOX-1 TG mice compared with WT mice. Medial wall thickness of the pulmonary arteries was significantly greater in LOX-1 TG mice than in WT mice. Furthermore, hypoxia enhanced ROS production and nitrotyrosine expression in LOX-1 TG mice, supporting the observed pathological changes. Administration of the NADPH oxidase inhibitor apocynin caused a significant reduction in RVSP and RV hypertrophy in LOX-1 TG mice compared with WT mice. Our results suggest that LOX-1-ROS generation induces the development and progression of PH.

pulmonary hypertension; oxidative stress; low-density lipoprotein receptor-1; NADPH oxidase; hypoxia

PULMONARY HYPERTENSION (PH) is characterized by a progressive increase in pulmonary vascular resistance and vascular remodeling, leading to right heart failure and death. Many vasoactive factors play important roles in the progression of PH, such as endothelin-1, prostanycin, and serotonin (5, 11, 19). Therapies have been developed to improve endothelial function, reduce pulmonary artery pressure, slow the progression of vascular remodeling in the pulmonary vasculature, and improve the patient’s clinical status and survival rate (33). Although interactions between genetic and environmental factors are considered to cause PH, its underlying causes are still largely unknown (10). Increased levels of ROS, an altered redox state, and elevated oxidant stress have been demonstrated in the lungs and right ventricle (RV) of several animal models of PH (8), including chronic hypoxia, monocrotaline toxicity, caveolin-1 knockout mice (46), and transgenic (TG) Ren2 rats, which overexpress the mouse renin gene (7). In our previous study (26), we demonstrated the effectiveness of hydroxytempol, a SOD mimetic, in a PH model of adrenomedullin knockout mice (26). However, the factor that stimulates ROS production in pulmonary vascular cells has yet to be identified.

Lectin-like oxidized low-density lipoprotein (oxLDL) receptor (LOX)-1 is a scavenger receptor for oxLDL and is primarily expressed in endothelial cells (37). OxLDL induces endothelium dysfunction and injury, and LOX-1 is therefore considered to be the key molecule causing oxLDL-induced endothelial dysfunction (28). The basal expression of LOX-1 in endothelial cells is very low in vitro, but it can be rapidly induced by proinflammatory, prooxidant, and mechanical stimuli such as oxLDL (20), ANG II (24), TNF-α (18), and shear stress (29). LOX-1 expression initiates endothelial nitric oxide (NO) synthase (eNOS) dysfunction (23) and reduces NO production (6). Furthermore, it promotes chemokine expression and monocye adhesion to endothelial cells (21). In vivo studies have suggested that LOX-1 might be a multipotent regulator of cardiovascular disease (4, 26, 30), such as atherosclerosis (3) and myocardial infarction (15), and may also be involved in balloon injury (12) and kidney injury (17). Inoue et al. (16) reported that after being placed on a high-fat diet for 3 wk, LOX-1 TG mice exhibited both accelerated intramyocardial vasculopathy and area enlargement with atheroma-like lesions compared with their non-TG littermates. Many studies have shown that LOX-1 activation initiates superoxide production by NADPH oxidase (41) and upregulation of various genes, including ROS-dependent signals (35), in endothelial cells. However, its role in the pulmonary vasculature has not yet been fully defined. Thus, in this study, we explored the hypothesis that overexpression of LOX-1 in the lungs and the consequent increase in oxidative stress promote the development of PH.

MATERIALS AND METHODS

Animals. LOX-1 TG mice were generated on a C57BL/6 background, as previously described (16). C57BL/6 (wild type [WT]) mice were purchased from Clea Japan (Tokyo, Japan). Male LOX-1 TG
and WT mice (10–12 wk old) were exposed to hypoxia as previously described (27). Mice were randomly divided into two groups. The first group was provided free access to drinking water, and the second group was provided drinking water treated with 5 mM apocynin (Alfa Aesar, Ward Hill, MA) from the start of the experiment until the day before hypoxic exposure. The hypoxic environment was maintained by a continuous mixed gas flow (10% O2-90% N2) at a rate of 500 ml/min). The chamber was opened for 5 min once every alternate day to clean the cages and replenish food and water supplies. Normoxic groups were kept on a wire rack adjacent to the chamber and exposed to room air. All mice were maintained at 26 ± 2°C with a 12:12-h light-dark cycle and were provided standard mouse chow and water ad libitum. All study protocols conformed with guiding principles of animal experimentation and were approved by the Ethics Committee on Animal Research of the University of Tokyo.

**Hemodynamic measurements.** Mice were anesthetized using 0.4 mg/g tribromoethanol with spontaneous breathing during the operation. Right ventricular (RV) systolic pressure (RVSP) was measured by retrograde insertion of a polyethylene tube (PE-10) into the right external jugular vein to reach the RV cavity, and data were acquired using a Power Lab system (AD Instruments, Colorado Springs, CO) and recorded on a computer using Chart software (AD Instruments).

**Quantification of pulmonary artery remodeling.** After 21 days of hypoxic and normoxic exposure, the lungs were excised, and 4% paraformaldehyde phosphate buffer solution (Wako, Tokyo, Japan) was injected into the trachea to expand the alveoli. The lungs were fixed using 4% paraformaldehyde, embedded in paraffin, and subsequently cut into 3-μm-thick sections, which were then stained with hematoxylin and eosin. Morphological changes in the small pulmonary arteries (external diameter: 60–100 μm) were evaluated as previously described (27). Wall thickness [percent mean thickness (%MT)] was calculated as follows: medial wall thickness (distance between the internal and external lamina) × 2/external diameter × 100. Images were obtained and analyzed using an Olympus FSX100 fluorescence microscope (Olympus, Tokyo, Japan). %MT was calculated for at least 10 small pulmonary arteries for each mouse, and the shortest external diameter was taken and measured to allow for vessels that were not perfectly symmetrical circles.

**Gravimetric assessment of RV and left ventricular hypertrophy.** Mouse ventricles were dissected so that they were free from the great vessels and atria, as previously described (38). The RV was isolated from the left ventricle (LV) and septum (S) by dissection under a microscope. RV and LV weights were expressed as the ratio of RV to LV + S weight [RV/(LV + S); Fulton’s ratio].

**ROS formation.** ROS formation was measured by chemiluminescence as previously described (27, 39). After the excision of lung tissues, samples were immediately homogenized and incubated in 1 ml HBSS buffer (Invitrogen) containing 10 mmol/l HEPES (Invitrogen) for 20 min at 37°C. After an incubation, lucigenin (Sigma-Aldrich, St. Louis, MO) was added to the mixture to obtain a final sample concentration of 100 μmol/l. Chemiluminescence was measured using a Lumat luminoimeter (LB 9507, Berthold Technologies, Bad Wildbad, Germany). Thereafter, NADPH (Sigma-Aldrich Japan, Tokyo, Japan) was added to the reaction mixture at a final concentration of 10 μmol/l just before luminescence measurements were initiated. The final measurement was obtained by subtracting the background count determined using identically treated tissue-free readings from the tissue readings. Samples were then normalized to total protein in the whole homogenate, and ROS values were expressed as counts per minute per milligram of protein. In some experiments, apocynin, which blocks NADPH oxidase, was used at a concentration of 100 μmol/l to confirm the generation of ROS from NADPH oxidase.

**NADPH oxidase activity.** NADPH oxidase activity was determined as previously described (7). Briefly, lung tissues were homogenized on ice in a homogenization buffer (50 mM phosphate buffer and 0.01 mM EDTA, pH 7.4) using a Daul homogenizer. The homogenate was centrifuged at 1,000 g for 30 min at 4°C, and the supernatant was further centrifuged at 13,000 g for 20 min at 4°C. Protein (100 μg) was incubated with NADPH (100 μM) at 37°C. NADPH oxidase activity was then determined by measuring the conversion of a radical detector (Cayman Chemical, Ann Arbor, MI) using a TECAN (TECAN Japan, Tokyo, Japan) at 450 nm every 10 min for 1 h. Immunochemistry. Nitrotyrosine was detected by fluorescence immunohistochemistry. Sections were incubated with Alexa fluor 488-conjugated goat anti-rabbit antibody (Invitrogen) to detect nitrotyrosine. Fluorescence intensity was quantified using image-analysis software (ImageJ, National Institutes of Health, Bethesda, MD). Proliferating cell nuclear antigen (PCNA) was examined as previously described (32). Lung tissue sections were stained with hematoxylin and anti-PCNA antibodies (BD Transduction Laboratories, San Diego, CA). Immunoreactivity was detected using an LSAB 2 Kit (Dako Japan, Tokyo, Japan) and visualized using 3,3′-diaminobenzidine substrate. A ×100 objective lens was used to photograph six random 0.5-mm² fields/genotype. All nuclei and PCNA-positive cells within these regions were manually tabulated using a hand-held counter.

**Statistical analysis.** All values were expressed as means ± SE. Comparisons among groups were made using one-way ANOVA and Tukey’s post hoc test. Statistical differences between only two groups were assessed using an unpaired Student’s t-test. P values of <0.05 were considered statistically significant. Statistical analyses were performed using Prism (Graphpad Software, San Diego, CA).

**RESULTS**

**PH increases in LOX-1 TG mice exposed to hypoxia.** To test whether overexpression of LOX-1 causes PH, we measured RVSP in LOX-1 TG and WT mice. Under normoxic conditions, RVSP was comparable between both LOX-1 TG and WT mice (Fig. 1A). However, after 3 wk of hypoxia, RVSP in LOX-1 TG mice was −1.86-fold higher than in normoxic LOX-1 TG mice and ~1.3-fold higher than in hypoxic WT mice (Fig. 1A). To determine the cause of PH, we examined pulmonary vascular remodeling. Under normoxic conditions, the normalized wall thickness of muscular arteries (diameter: 60–100 μm) did not differ between LOX-1 TG and WT mice (14.55 ± 1.63% and 17.68 ± 2.71%, respectively). However, LOX-1 TG mice showed significantly higher vessel wall thickness than WT mice. Furthermore, %MT was significantly higher in LOX-1 TG mice than in WT mice (15.62 ± 1.92% and 22.93 ± 1.65%, respectively, P < 0.05; Fig. 1B). Histochemical analysis of the heart revealed results consistent with RV hypertrophy and revealed that under hypoxic conditions, the RV wall mass was higher in LOX-1 TG mice. While hypoxia induced significant RV hypertrophy in LOX-1 TG mice (Fig. 1C), there were no differences in values of RV hypertrophy, as measured by Fulton’s index [RV/(LV + S)] and absolute RV weights between the two groups of mice under normoxic conditions (Fig. 1C).

**Oxidative stress is associated with more severe PH in LOX-1 TG mice.** The observed increase in ROS production could be due to increased oxidase-specific activity, upregulation of enzyme levels, or stimulation of activation factors. We used lucigenin-enhanced chemiluminescence to identify the factor that increased ROS production and the tetrazolium salt method to quantify NADPH oxidase activity. Under normoxic conditions, ROS production and NADPH activity were similar between the two groups of mice; however, under hypoxic conditions, they were significantly higher in LOX-1 TG mice than in WT mice (Fig. 2, A and B). NO is a product of the NO synthase-catalyzed conversion of arginine to citrulline, and it
reacts rapidly with superoxide to form peroxynitrite. Previously, it has been reported that nitrotyrosine on proteins can serve as a marker for peroxynitrite formation in vivo (27). Under normoxic conditions, nitrotyrosine levels were similar between both groups of mice. However, under hypoxic conditions, nitrotyrosine levels in lung sections were markedly higher in LOX-1 TG mice, whereas there was a modest increase in nitrotyrosine levels of WT mice (Fig. 2C). We

Fig. 1. A: representative right ventricular (RV) systolic pressure (RVSP) tracings of wild-type (WT) and lectin-like oxidized low-density lipoprotein receptor (LOX)-1 transgenic (TG) mice. Mice were exposed to normoxic (N) and hypoxic (H) conditions for 3 wk (n = 5–6 animals/group). Values are expressed as means ± SE. B: quantification of the percent wall thickness [percent mean thickness (%MT)] of pulmonary arterioles in the lungs of WT and TG mice after exposure to normoxic and hypoxic conditions (n = 6 mice/group). Ten vessels were analyzed per mouse. Data are expressed as means ± SE. C: representative RV hypertrophy in normoxia and after hypoxia in WT and TG mice. The ratio of the RV to the left ventricle plus septum [RV/(LV + S), n = 6 animals/group] was obtained. Values are expressed as mean ± SEM.
evaluated cell proliferation in lung tissues of mice from both groups under hypoxic conditions. The ratio of PCNA-positive cells to total cells in lung tissue was substantially increased in LOX-1 TG mice compared with WT mice (Fig. 2D).

Effects of antioxidants on PH in LOX-1 TG mice. To determine whether antioxidants attenuate PH in LOX-1 TG mice exposed to hypoxia, we examined whether treatment with NAPDH oxidase inhibitor could reverse PH in LOX-1 TG mice. Apocynin inhibits NAPDH oxidase, preventing the generation of ROS from NAPDH oxidase. Apocynin treatment resulted in lower RVSP and vascular remodeling in LOX-1 TG mice (Fig. 3). ROS production and NADPH oxidase activity were reduced with apocynin adminis-

Fig. 2. A: ROS production was measured by lucigenin-enhanced chemiluminescence. Sections from one whole murine lung (100 mg) were homogenized and incubated with 10 μmol/l NADPH oxidase. Values are expressed as means ± SE. RLU, relative light units. B: NADPH oxidase activity was measured using tetrazolium salt. Values are expressed as means ± SE. C: immunofluoresstaining for the detection of nitrotyrosine. Magnification: ×100. Fluorescence intensity in mouse lung sections was quantified using ImageJ software. Values are expressed as means ± SE. D: representative histological sections from the lungs of normoxic and hypoxic WT and TG mice were stained for proliferating cell nuclear antigen (PCNA). The arrowheads indicate PCNA-positive cells. Quantitative analysis of the ratio of PCNA-positive cells to the total cell number in the lung is shown (n = 6–7). Values are expressed as means ± SE.
DISCUSSION

We (27) previously reported that chronic hypoxia increases oxidant stress and vascular remodeling in lungs and that treatment with SOD blocked hypoxia-induced vascular remodeling. In this study, we showed that overexpression of LOX-1 increased RVSP and ROS generation under hypoxic conditions and that apocynin treatment reversed PH and nitrotyrosine expression in the pulmonary vasculature in hypoxic LOX-1 TG mice (Fig. 5). These data indicated that the activation of NADPH oxidase by LOX-1 plays an important role in the pathogenesis of PH.

LOX-1 has been demonstrated to actively contribute to several vascular diseases, including myocardial infarction (22), heart failure (44), and kidney injury (17). LOX-1 is expressed not only in endothelial cells but also in macrophages, vascular smooth muscle cells, and platelets. The baseline expression of LOX-1 in endothelial cells is minimal; however, it can be rapidly induced by proinflammatory, prooxidative, and mechanical stimuli. Correspondingly, LOX-1 can be enhanced by several pathological conditions, including hypertension, diabetes mellitus, hyperlipidemia, and chronic renal failure. An endothelial-specific LOX-1 overexpression model was developed to study the contribution of the LOX-1 receptor, and it showed coronary vasculopathy in animals fed a high-fat diet (16). 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine percholate-labeled oxLDL uptake was enhanced in endothelial cells, and oxidative stress was increased in the heart of LOX TG mice. It is known that the pattern of transgene expression in LOX-1 TG mice is similar to that in non-TG mice and demonstrates tissue-specific and ontogenetic regulation (16). Pathological and physiological comparisons of WT and LOX-1 TG mice of the same age maintained under normoxic conditions revealed few significant differences.

In our study, ROS production and RV pressure were significantly increased in LOX-1 TG mice under hypoxic conditions (Figs. 1 and 2). Moreover, pulmonary vascular remodeling and PH resulted in RV hypertrophy in LOX-1 TG mice after exposure to hypoxia (Fig. 1). This suggests that the overexpression of LOX-1 requires the presence of its receptor and ligands and that ROS production by LOX-1 depends on the ligands binding to it under hypoxic conditions. In addition to oxLDL, LOX-1 binds to other ligands (2, 37), including aged cells, apoptotic cells (31), C-reactive protein (40), platelets (36), and leukocytes (13). LDL oxidation has been reported to increase in hypoxia (34), and other ligands have been suspected to increase in hypoxic conditions. However, the question of which LOX-1 ligands are elevated in hypoxic mice models remains unanswered. It has been reported that mitochondrial superoxide plays an important role in PH development (1); our preliminary data suggest that mitochondria-derived ROS may play a minor role in our model (data not shown) and that NADPH oxidase plays a pivotal role.

In the presence of superoxide, NO produces peroxynitrite, which modifies proteins and interferes with NO function. Chronic hypoxia leads to both increased superoxide levels and impaired NO production, despite increased eNOS expression. Our previous studies have shown that peroxynitrite expression is elevated in hypoxic lungs and that LOX-1 increases intracellular ROS production and eNOS phosphorylation in vitro (35). The data from our present study showed that peroxynitrite formation was markedly increased in LOX-1 TG hypoxic lungs, as evidenced by the increased nitrotyrosine levels by immunostaining (Fig. 2). LOX-1 binding to oxLDL enhanced the catabolism of NO by superoxide generation and decreased NO release via attenuated eNOS activity (43). The imbalance of NO and oxidative stress resulting from the binding of ligands to LOX-1 causes endothelial dysfunction. It has been...
reported caveolin knockout mice develop PH and RV hypertrophy in association with pulmonary vascular remodeling, hypercellularity, and alveolar septal thickening (9). Nitrotyrosine binds to PKG and impairs its activity, leading to vasoconstriction and pulmonary vascular remodeling in caveolin knockout mice (46). It has also been reported that scavenging of superoxide by manganese (III) tetrakis (1-methyl-4-pyridyl) porphyrin pentachloride (a SOD mimetic) could reverse PH in caveolin knockout mice. In the present study, we did not check PKG activity and nitrosylation, but we suggest that PKG activity might be impaired, and this might influence PH in LOX-1 TG mice after hypoxia.

Apocynin has been used as an efficient inhibitor of the NADPH oxidase complex in many experimental models. It
pulmonary vascular disease. LOX-1 itself binds to monocytes and activates superoxide generation in endothelial cells (35), indicating that apocynin plays a protective role in attenuating free radicals by LOX-1 activation after endothelial cells (35), indicating that apocynin possibly inhibits peroxynitrite formation, which could be potentially useful for the treatment of respiratory disorders. LOX-1 affects lung tissue, and its expression may contribute to the pathogenesis of pulmonary vascular disease. We believe that the results of our study provide a further rationale for investigating the role of LOX-1 in animal models of PH and in patients with pulmonary vascular disease.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: S.O., T. Shimosawa, M.-S., and T.F. conceived and designed of research; S.O., S.M., and T. Sonobe performed experiments; S.O. analyzed data; S.O., T. Shimosawa, H.W., K.-i.Y., Y.Y., M.S., and T.F. interpreted results of experiments; S.O. prepared figures; S.O. drafted manuscript; S.O., F.K.-M., H.W., K.-i.Y., Y.U., K.-i.Y., Y.Y., and M.S. edited and revised manuscript; S.O., T. Shimosawa, K.-i.Y., and Y.Y. approved final version of manuscript.

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