The Nuclear Factor Erythroid 2–Related Factor 2 Activator Oltipraz Attenuates Chronic Hypoxia–Induced Cardiopulmonary Alterations in Mice

Shunsuke Eba1, Yasushi Hoshikawa1, Takashi Moriguchi2, Yoichiro Mitsuishi2, Hironori Satoh2, Kazuyuki Ishida3, Tatsuaki Watanabe1, Toru Shimizu4, Hiroaki Shimokawa4, Yoshinori Okada1, Masayuki Yamamoto2, and Takashi Kondo1

1Department of Thoracic Surgery, Institute of Development, Aging and Cancer, Tohoku University; 2Department of Medical Biochemistry, Tohoku University Graduate School of Medicine; 3Department of Diagnostic Pathology, Iwate Medical University School of Medicine; and 4Department of Cardiovascular Medicine, Tohoku University Graduate School of Medicine, Sendai, Japan

Nuclear factor erythroid 2–related factor 2 (Nrf2) is a key regulator that activates many antioxidant enzymes. Oxidative stress, which accumulates in diseased lungs associated with pulmonary hypertension (PH), is thought to be responsible for the progression of cardiopulmonary changes. To test whether Nrf2 activation would exert therapeutic efficacy against cardiopulmonary changes in a hypoxia-induced PH model, wild-type (WT) and Nrf2-deficient mice as well as Kelch-like ECH associated protein 1 (Keap1) (negative regulator of Nrf2) knockout mutant mice were exposed to hypobaric hypoxia for 3 weeks. This chronic hypoxia exacerbated right ventricular systolic pressure, right ventricular hypertrophy (RVH), and pulmonary vascular remodeling in the WT mice. These pathological changes were associated with aberrant accumulation of Tenascin-C, a disease-indicative extracellular glycoprotein. Simultaneous administration of oltipraz, a potent Nrf2 activator, significantly attenuated RVH and pulmonary vascular remodeling and concomitantly ameliorated Tenascin-C accumulation in the hypoxic mice. Hypoxia-exposed Nrf2-deficient mice developed more pronounced RVH than WT mice, whereas hypoxia-exposed Keap1-knockdown mice showed less RVH and pulmonary vascular remodeling than WT mice, underscoring the beneficial potency of Nrf2 activity against PH. We also demonstrated that expression of the Nrf2-regulated antioxidant enzymes was decreased in a patient with chronic obstructive pulmonary disease associated with PH. The decreased antioxidant enzymes may underlie the pathogenesis of cardiopulmonary changes in the patient with chronic obstructive pulmonary disease and PH. The pharmacologically or genetically induced Nrf2 activity clearly decreased RVH and pulmonary vascular remodeling in the hypoxic PH model. The efficacy of oltipraz highlights a promising therapeutic potency of Nrf2 activators for the prevention of PH in patients with hypoxic lung disease.

Keywords: Nrf2; Keap1; pulmonary vascular remodeling; chronic hypoxia; reactive oxygen species

Chronic hypoxia represents a pathophysiological insult; it induces pulmonary hypertension (PH), which is characterized by right ventricular hypertrophy (RVH) and pulmonary vascular remodeling in humans and animal models at higher altitudes (1–4). These pathophysiological changes to the cardiopulmonary system have been deemed critical exacerbating factors that determine the prognosis of patients suffering from various respiratory diseases presenting with hypoxemia, including chronic obstructive pulmonary disease (COPD) and idiopathic pulmonary fibrosis (IPF) (5, 6). Therefore, intense efforts have been exerted to elucidate the etiological basis of PH in the hopes of finding novel therapeutic targets. Exposure to hypoxic stress has been shown to induce overproduction of reactive oxygen species (ROS) in multiple animal models (7, 8), which leads to the progression of pulmonary vascular remodeling by up-regulating several humoral and intracellular signaling factors that induce vascular remodeling, namely vascular endothelial growth factor, platelet-activating factor, and mitogen-activated protein kinase, in pulmonary arterial endothelial and smooth muscle cells (9–14). Several years ago, we demonstrated that simultaneous treatment with antioxidant N-acetylcysteine dramatically attenuates the pathophysiological changes induced by chronic hypoxic exposure in rats (7). The therapeutic effect of N-acetylcysteine treatment is associated with significant decreases in the phosphatidylcholine hydroperoxide level in the lung tissue (7). These results indicate that the accumulation of ROS induced by chronic exposure to hypoxia probably contributes to the pathogenesis and progression of pulmonary vascular remodeling and the subsequent occurrence of PH. Nuclear factor erythroid 2–related factor 2 (Nrf2) is a basic region leucine zipper transcription factor that serves as a key regulator of cellular defense against oxidative stress (15). In the absence of oxidative stress stimuli, Nrf2 is rapidly degraded by the ubiquitin–proteasome pathway through association with Kelch-like ECH associating protein 1 (Keap1), a cullin3-based ubiquitin E3 ligase (16, 17). Upon exposure to oxidative stress, Keap1 is inactivated through chemical modification of its cysteine residues. Nrf2 is thereby liberated from Keap1-mediated degradation and subsequently translocates to the nucleus, where it dimerizes with a member of the small Maf family of proteins. This heterodimerized complex activates the transcription of a wide range of antioxidant genes, including NAD phosphate reduced quinone oxidoreductase (NQO1), glutathione S-transferase, glutamate–cysteine ligase catalytic subunit (GCLC), and hemeoxygenase-1 (HO-1) via a cis-acting DNA element known as antioxidant responsive element. Thus, the molecular basis of the Nrf2-mediated antioxidative stress response is attributed to the increased transcription of antioxidant enzyme genes that accelerate the clearance of ROS-mediated cellular damage (15).

Given the central role of Nrf2 in the antioxidant response, we hypothesized that pharmacological activation of the Nrf2 pathway might exert therapeutic efficacy in a chronic hypoxia–induced murine PH model. The results of this study were consistent with this hypothesis. Chronic administration of the highly potent Nrf2 activator oltipraz significantly decreased pulmonary vascular remodeling and RVH associated with chronic hypoxia.
hypoxia–induced PH. This favorable therapeutic effect exerted by oltipraz administration was not observed in the Nrf2-deficient (Nrf2–/–) mice, indicating that the efficacy of oltipraz was highly dependent on Nrf2 activity. Moreover, we demonstrated that chronic hypoxia–induced pulmonary vascular remodeling and RVH were significantly attenuated in Keap1 knockout (Keap1f/f) mice in which the nuclear Nrf2 protein is constitutively accumulated because of the decreased Keap1 expression (18).

Tenascin-C (TN-C) is a representative extracellular matrix glycoprotein, the expression level of which is known to be associated with the disease progression of PH (19, 20). It has been reported that the bleomycin-induced lung fibrosis model abundantly accumulated TN-C in Nrf2–/– mice compared with Nrf2+/+ mice (21), indicating that Nrf2 activity plays an important role in reducing TN-C expression. Indeed, lung TN-C expression was increased in Nrf2–/– mice after 1 week of hypoxic exposure, whereas this hypoxia-induced increase in TN-C expression was significantly reduced by the oltipraz treatment.

In summary, these results strongly suggest that Nrf2 plays a crucial physiological role in the modulation of the cardiopulmonary response to chronic hypoxia. The efficacy of oltipraz highlights the promising therapeutic potency of Nrf2 activators for the prevention of PH associated with severe respiratory diseases presenting with hypoxemia.

MATERIALS AND METHODS

Further details are provided in the online supplement.

Animals and Treatments

Nrf2–/– and Keap1f/f mice backcrossed to a C57BL/6J genetic background were used in this study (18, 22). Mice were matched to wild-type (WT) (Nrf2+/+) control mice by weight and age. The mice were exposed to hypoxia using a hypobaric chamber (Shizume Medical, Tokyo, Japan) at a simulated altitude of 17,000 feet as described previously (23). The mice were treated with oltipraz (5, 50, or 500 mg/kg in 0.1 ml of 1% cremophor and 25% glycerol; LKT Laboratories, Inc., St. Paul, MN) by gavage or vehicle every 24 hours. All protocols were approved by the Tohoku University Animal Care Committee (Sendai, Japan).

Immunoblotting Analyses

Nuclear extracts were prepared from the left lungs of the mice using the NE-PER Nuclear and Cytoplasmic Extraction Reagent kit (Thermo Scientific, Waltham, MA). Immunoblotting analysis was performed using anti-Nrf2 and anti-Lamin B antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) as reported previously (16).

Real-Time qRT-PCR

Total RNA was extracted from lung tissue using ISOGEN (Nippon Gene, Toyama, Japan). qRT-PCR was performed using an ABI PRISM 7000 sequence detector system (PE-Applied Biosystems, Foster City, CA) and SYBR Premix Ex Taq II (TaKaRa, Shiga, Japan) as described previously (18). The primer sequences for Nrf2, NQO1, GCLC, HO-1, TN-C, β-actin, and 18 s rRNA are shown in Table E1 in the online supplement.

Measurement of Right Ventricular Systolic Pressure and Right Ventricular Hypertrophy

Right ventricular systolic pressure (RVSP) was measured by a direct puncture method as described previously (24). The weight of the right ventricular (RV) free wall and that of the left ventricle and septum (LV + S) were measured separately, and their ratio, RV/(LV + S), was calculated to estimate RVH (24).

Immunohistochemistry

The right lungs of the mice were fixed in an inflated state with 10% buffered formaldehyde solution, and paraffin sections were processed for immunostaining. Mouse anti-α smooth muscle actin (α-SMA) antibody (DAKO, Glostrup, Denmark) and goat anti–Tenascin-C antibody (Santa Cruz Biotechnology) were used. For visualization, tissue sections were incubated with horseradish peroxidase-conjugated antirabbit secondary antibody (Invitrogen, Carlsbad, CA) and detected using the avidin-biotin-peroxidase system (Nichirei Corp., Tokyo, Japan).

Morphometric Analysis of the Pulmonary Arteries

Hilar sections of each right lung were subjected to α-SMA immunohistochemistry analysis and microscopically assessed for remodeling of pulmonary arteries (PAs) as described previously (23, 24).

Flow Cytometry

Single-cell suspensions of lung tissue were prepared as previously described (25) for ROS quantification by flow cytometry using 2′,7′-dichlorodihydrofluoresceindiacetate (DCFDA) (Invitrogen) fluorescence and qRT-PCR of antioxidant genes.

Statistical Analyses

All values are expressed as mean ± SEM. Comparisons were made using one-way ANOVA with Tukey’s multiple comparison and unpaired t tests. A P value of < 0.05 was considered statistically significant.

RESULTS

Oltipraz Treatment Induces the Nrf2 Regulatory Pathway in Lung Tissue

Immunoblot analysis using whole lung nuclear extracts showed significantly higher nuclear accumulation of Nrf2 protein after oltipraz treatment (50 mg/kg/d for 3 d) in Nrf2–/– mice (*P < 0.05 versus vehicle-treated Nrf2+/+ mice) (Figures 1A [left panel] and 1B). In contrast, Nrf2 accumulation was virtually absent in the lungs of Nrf2–/– mice even with oltipraz (500 mg/kg) treatment (Figure 1A). Vehicle-treated Keap1f/f mice showed the abundant accumulation of Nrf2 protein, reaching a level almost equivalent to that in Nrf2+/+ mice treated with 500 mg/kg (Figures 1A [right panel] and 1B). Oltipraz treatment (500 mg/kg) rarely induced an additional increase in nuclear Nrf2 protein in Keap1f/f mice (Figures 1A [right panel] and 1B).

Despite the accumulated Nrf2 protein, qRT-PCR showed that Nrf2 mRNA expression level in the lung was not substantially changed at 24 hours after a single administration of oltipraz (5, 50, or 500 mg/kg) in Nrf2–/– mice (Figure 1C). As expected, Nrf2 mRNA expression was not detected in the lungs of Nrf2–/– mice regardless of oltipraz treatment (Figure 1C). The mRNA expression of NQO1 and GCLC in the lungs of Nrf2–/– mice was induced by oltipraz treatment in a dose-dependent manner (Figures 1D and 1E).

In contrast, inducible expression of these genes was barely detectable in Nrf2–/– mice administered oltipraz at 500 mg/kg. After a single administration of oltipraz (50 mg/kg) to Nrf2–/– mice (Figure 1C). As expected, Nrf2 mRNA expression was not detected in the lungs of Nrf2–/– mice regardless of oltipraz treatment (Figure 1C). The mRNA expression of NQO1 and GCLC in the lungs of Nrf2–/– mice was induced by oltipraz treatment in a dose-dependent manner (Figures 1D and 1E). In contrast, inducible expression of these genes was barely detectable in Nrf2–/– mice administered oltipraz at 500 mg/kg. After a single administration of oltipraz (50 mg/kg) to Nrf2–/– mice (Figure 1C). As expected, Nrf2 mRNA expression was not detected in the lungs of Nrf2–/– mice regardless of oltipraz treatment (Figure 1C).

Chronic Hypoxia-Induced Cardiopulmonary Changes Are Attenuated by Oltipraz Administration: Pharmacological Activation of Nrf2

To test the therapeutic efficacy of oltipraz on cardiopulmonary pathogenesis associated with chronic hypoxia–induced PH, we...
exposed Nrf2+/+ mice to hypobaric hypoxia for 3 weeks and simultaneously administered vehicle or 50 mg/kg of oltipraz 5 days a week for 3 weeks. The doses and frequency of oltipraz administration were determined on the basis of the efficient inducibility of NQO1 and GCLC mRNA expression at 24 hours after administration of oltipraz (50 mg/kg) (Figures 1D–1G). Changing the dose and frequency of oltipraz to 500 mg/kg once a week resulted in almost similar inhibitory effects in the following experiments (data not shown) (26). Mice subjected to the hypoxic exposure showed barely any gains in body weight, whereas age-matched control mice exposed to normoxic conditions showed normal weight gain regardless of oltipraz administration (see Figure E1 in the online supplement). Hematocrit levels were increased up to 60% in the hypoxic mice, whereas those of normoxic mice were around 40% on average (Table E2). Oltipraz administration had no effect on hematocrit levels in normoxia or hypoxia (Table E2).

Three weeks of hypoxic exposure led to the development of PH (i.e., an increase in RVSP values) and RVH (i.e., an increase in the RV/[LV + S] ratio) in vehicle-treated Nrf2−/− mice (Table 1). Oltipraz administration significantly decreased hypoxia-induced RVH, whereas this medication exerted no effect on the increased RVSP in hypoxic Nrf2−/− mice, indicating that pharmacological activation of Nrf2 had a pronounced effect in reducing hypoxia-associated RVH without reducing RVSP (Table 1). Under normoxic conditions, oltipraz had no effect on RVSP or the RV/[LV + S] ratio in Nrf2−/− mice (Table 1).

To evaluate muscularization of the small pulmonary arterioles around the level of the alveolar duct, the number of nonmuscular, partially muscular, and fully muscular vessels was counted in lung sections from each mouse by means of immunohistochemistry for
α-SMA (see the online supplement for categorization). More than 80% of pulmonary arterioles in the vehicle-treated normoxic Nrf2+/−/+ mice exhibited nonmuscular vessels, whereas the remaining 19% of arterioles had partially (17.0%) or fully (2.2%) muscular walls. In contrast, in hypoxic Nrf2+/−/+ mice, the number of partially or fully muscular pulmonary arterioles was significantly increased to 39.4 and 9.2%, respectively. The proportion of nonmuscular pulmonary arterioles was decreased to 51.4% (Table 1). Oltipraz administration significantly decreased the muscularization of the pulmonary arterioles in hypoxic Nrf2+/−/+ mice. The proportion of nonmuscular vessels recovered to 62.4%, and that of partially and fully muscular pulmonary arterioles decreased to 32.8 and 4.8%, respectively, in oltipraz-treated hypoxic Nrf2+/−/+ mice. In addition, chronically hypoxic Nrf2+/−/+ mice showed increased vessel wall thickness (VWT) of the PAs at the level of the terminal bronchiolte (TB) when compared with normoxic Nrf2+/−/+ mice (Table 1). As expected, VWT of the oltipraz-treated hypoxic mice was significantly lower than that of the vehicle-treated hypoxic mice (Table 1). In summary, oltipraz administration significantly attenuated the pathogenic muscularization of PA, which presumably contributed to the improvement in RVH under chronic hypoxia. In contrast, oltipraz treatment had no effect on elevated RVSP.

### TABLE 2. EFFECTS OF NRF2 KNOCKOUT ON HYPOXIA-INDUCED PULMONARY HYPERTENSION, RIGHT VENTRICULAR HYPERTROPHY, MUSCULARIZATION OF SMALL PULMONARY ARTERIOLES, AND VESSEL WALL THICKNESS OF THE PULMONARY ARTERIES AFTER 3 WEEKS OF EXPOSURE TO HYPOXIA

<table>
<thead>
<tr>
<th>Measurements</th>
<th>Nrf2+/−/+</th>
<th>Nrf2−/−</th>
<th>Nrf2+/−/+</th>
<th>Nrf2−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>RVSP, mm Hg</td>
<td>(24.4 ± 0.7 (13)*</td>
<td>22.2 ± 0.9 (11)</td>
<td>34.8 ± 1.1† (17)</td>
<td>29.2 ± 1.0† (12)</td>
</tr>
<tr>
<td>RV/(LV + S)</td>
<td>0.23 ± 0.01 (9)</td>
<td>0.28 ± 0.02 (4)</td>
<td>0.40 ± 0.01† (17)</td>
<td>0.467 ± 0.01† (12)</td>
</tr>
<tr>
<td>Muscularization of small PA</td>
<td>(81.8 ± 0.6)</td>
<td>(78.8 ± 0.5)</td>
<td>(54.7 ± 1.4†)</td>
<td>(50.9 ± 2.6†)</td>
</tr>
<tr>
<td>Nonmuscular, %</td>
<td>17.0 ± 0.7</td>
<td>19.0 ± 0.4</td>
<td>37.6 ± 0.9†</td>
<td>43.9 ± 2.7†</td>
</tr>
<tr>
<td>Fully muscular, %</td>
<td>1.3 ± 0.3</td>
<td>2.3 ± 0.5</td>
<td>7.7 ± 2.5†</td>
<td>5.3 ± 0.8†</td>
</tr>
<tr>
<td>VWT of PAs</td>
<td>0.214 ± 0.005 (4)</td>
<td>0.209 ± 0.006 (4)</td>
<td>0.262 ± 0.005 (4)</td>
<td>0.248 ± 0.006 (4)</td>
</tr>
</tbody>
</table>

Definition of abbreviations: Nrf2+/−/+ = wild-type mice; Nrf2−/− = Nrf2 knockout mice; RVSP = right ventricular systolic pressure; RV/(LV + S) = ratio of the right ventricular free wall to the combined weight of the left ventricle + septum; RVH = right ventricular hypertrophy; VWT = vessel wall thickness (external diameter of PA − internal diameter)/external diameter).

* Values are mean ± SE. Number of mice is indicated in parentheses.
† P < 0.05 versus Nrf2+/−/+ normoxia.
‡ P < 0.05 versus Nrf2+/−/+ hypoxia.
between hypoxia-exposed partially muscular, and fully muscularized small arterioles did not differ similar after chronic hypoxic exposure.

Nrf2 analysis of vessels in the lung sections of mice used for immunohistochemistry the number of nonmuscular, partially muscular, and fully muscular larization of the small pulmonary arterioles was evaluated by counting tricle the right ventricular free wall to the combined weight of the left ven-

Increased ROS Accumulation in CD31+ Pulmonary Vascular Endothelial Cells upon Hypoxic Exposure

We next tried to quantitatively assess ROS accumulation using DCFDA (a fluorescent ROS indicator) in CD31+ pulmonary vascular endothelial cells (PVECs). Expectedly, the DCFDA level in lung CD31+ cells from Nrf2+/− mice after 2 days of hypoxia exposure was significantly higher than that from normoxic Nrf2+/− mice (Figures 3A and 3B). Furthermore, hypoxia-exposed Nrf2+/− mice showed higher ROS accumulation in lung CD31+ cells than hypoxia-exposed Nrf2+/− mice. Lung CD31+ cells in Nrf2−/− mice displayed a slightly higher DCFDA levels than those in Nrf2+/− mice even under normoxia, suggesting a potential role of Nrf2 for ROS clearance in normoxic pulmonary endothelial cells (Figures 3A and 3B).

Antioxidant Genes Are Differentially Expressed in CD31+ PVECs after Hypoxic Exposure

Given the robustly increased ROS accumulation in CD31+ PVECs observed on hypoxic exposure, we examined the inducible expression of Nrf2 target genes. First, we evaluated overall changes in the expression of NQO1 and GCLC in lungs of hypoxia-exposed WT mice by qRT-PCR and immunohistochemistry. Despite increased ROS in the PVECs, NQO1 and GCLC mRNA were decreased in hypoxic WT mice when compared with normoxic WT mice (Figures E3A and E3B). Immunohistochemical analysis revealed that NQO1 was mainly expressed in the PA media (Figures E3C and E3D, arrows), whereas GCLC expression was preferentially observed in the endothelium (Figures E3G and E3H, arrows). After exposure to hypoxia for 2 days, the NQO1 (Figures E3E and E3F) and GCLC (Figures E3I and E3J) signals became barely detectable in the PA.

To further explore antioxidant gene expression at the cellular level, we examined mRNA expression of Nrf2 and its target genes in the CD31+ endothelial fraction sorted from the lungs of WT mice upon 2 days of hypoxia exposure. Nrf2 mRNA expression levels were not significantly different between the hypoxic and normoxic conditions (Figure 3C). Changes in the NQO1 mRNA levels in the CD31+ cells did not reach statistically significance after 2 days of hypoxia exposure (Figure 3D). In contrast, mRNA expression of GCLC and HO-1 was decreased in the lung CD31+ cells of WT mice after 2 days of hypoxia exposure (Figures 3E and 3F). WT and Nrf2−/− mice commonly exhibited low levels of GCLC and HO-1 mRNA expression in lung CD31+ cells under hypoxia (Figures 3E and 3F).

Hypoxia-Induced Pulmonary TN-C Expression Is Attenuated by Oltipraz treatment

To explore the pathological mechanism by which oltipraz decreases the hypoxia-induced pulmonary vascular remodeling in mice, we examined the effect of oltipraz treatment on expression of TN-C, which is an extracellular matrix protein associated...
with progression of PH (19, 20). TN-C mRNA abundance in WT mice was significantly increased after 7 days of exposure to hypoxia, as we previously reported (20). Oltipraz treatment attenuated the increased TN-C mRNA levels in hypoxic WT mice (Figure 4A). Concomitantly, immunohistochemical analyses with anti-TN-C antibody demonstrated an increased intensity of TN-C signals in the external elastic lamina of the PA walls in hypoxic WT mice (Figure 4C, arrows) compared with normoxic WT mice (Figure 4B). In contrast, the oltipraz administration decreased the TN-C immunoreactivity in the PA walls (Figure 4D, arrowheads). Closer examination of lung sections from vehicle-treated hypoxic WT mice revealed that the induced TN-C immunoreactivity was predominantly localized to the PA walls, as shown by the level of the TB (Figures 4E–4G, arrows) rather than the level of alveolar duct (Figures 4H and 4I, arrowheads). This observation was consistent with the increased VWT of PA in the hypoxic mice, which was detected predominantly around the level of TB (Table 1). Therefore, the oltipraz-mediated improvement in PA wall thickening could be attributed to the decreased TN-C expression in the PA walls around the level of TB.

### DISCUSSION

The principal findings of this study are as follows: 1) Gavage administration of oltipraz, a potent Nrf2 activator, enhanced the expression level of representative antioxidant genes NQO1 and GCLC by inducing nuclear accumulation of Nrf2 protein in mouse lung tissues; 2) treatment with oltipraz significantly attenuated RVH and pulmonary vascular remodeling associated with the accumulation of TN-C developed after 3-week exposure to hypobaric hypoxia in WT mice; 3) the favorable therapeutic effect of oltipraz on hypoxia-induced cardiopulmonary changes was not observed in Nrf2−/− mice; and 4) Keap1f/f mice showed increased nuclear Nrf2 protein and developed significantly less RVH and pulmonary vascular remodeling after chronic exposure to hypobaric hypoxia compared with WT mice. Collectively, our data strongly indicated that Nrf2 played an important role in the improvement in cardiopulmonary alterations in a hypoxic PH model and that Nrf2 activators may prove to be useful therapies for the PH that often accompanies hypoxic lung diseases like IPF and COPD.

The Nrf2 activator oltipraz is a substituted dithiolthione that is structurally related to similar chemical groups found in cruciferous vegetables (28). Consumption of these vegetables has been associated with a decreased human cancer risk (28). In- deed, oltipraz has been reported as having chemopreventive activity in a broad spectrum of animal models of carcinogenesis (29–31). The chemopreventive efficacy of oltipraz against gastric neoplasia in benzo[a]pyrene-treated mice was completely inhibited in Nrf2−/− mice (32). Thereafter, the efficacy of oltipraz was mediated by antioxidant and detoxifying gene expression through Nrf2 activation (32). Modification of critical cysteine residues of Keap1 provides a fundamental mechanism for Nrf2 activation (16, 17). On oxidation of Keap1 cysteine residues, conformational changes led to nuclear Nrf2 accumulation, thereby inducing antioxidant responsive element–mediated transcriptional activation (16, 17). Although a direct reaction of oltipraz with cysteine residues in Keap1 has remained elusive, oltipraz is known to be reactive with thiol groups (33). Through examination of the responsiveness of Keap1 cysteine substitution mutants to oltipraz treatment, we should be able to elucidate the detailed mechanism of oltipraz activity. Oltipraz induces nuclear Nrf2 protein accumulation and expression of Nrf2 target genes in mouse liver (34) and adipose tissue (35). However, whether oltipraz induces Nrf2 activation in the lung remains uncertain. We demonstrated that gavage administration of oltipraz increased Nrf2 nuclear accumulation in lung tissues of WT mice, although the Nrf2 mRNA expression level was not substantially changed. Meanwhile, NQO1 and GCLC expression was clearly induced by oltipraz

### TABLE 3. EFFECTS OF KELCH-LIKE ECH ASSOCIATING PROTEIN 1 KNOCKDOWN ON HYPOXIA-INDUCED PULMONARY HYPERTENSION, RIGHT VENTRICULAR HYPERTROPHY, MUSCULARIZATION OF SMALL PULMONARY ARTERIOLES, AND VESSEL WALL THICKNESS OF THE PULMONARY ARTERIES AFTER 3 WEEKS OF EXPOSURE TO HYPOXIA

<table>
<thead>
<tr>
<th>Measurements</th>
<th>Normoxia</th>
<th>Keap1f/f</th>
<th>Hypoxia</th>
<th>Keap1f/f</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild-type</td>
<td>Keap1f/f</td>
<td>Wild-type</td>
<td>Keap1f/f</td>
</tr>
<tr>
<td>RVSP (mmHg)</td>
<td>20.8±1.2 (4)*</td>
<td>23.4±0.4 (4)</td>
<td>33.3±0.9† (10)</td>
<td>31.2±1.3‡ (8)</td>
</tr>
<tr>
<td>RV/(LV + S)</td>
<td>0.22±0.01 (4)</td>
<td>0.27±0.01 (4)</td>
<td>0.43±0.01† (10)</td>
<td>0.34±0.01† (8)</td>
</tr>
<tr>
<td>Muscularization of small PA</td>
<td>(4)</td>
<td>(4)</td>
<td>(4)</td>
<td>(4)</td>
</tr>
<tr>
<td>Nonmuscular, %</td>
<td>80.0±1.6</td>
<td>80.0±1.2</td>
<td>46.5±1.0†</td>
<td>59.5±0.9‡</td>
</tr>
<tr>
<td>Partially muscular, %</td>
<td>19.8±1.7</td>
<td>17.8±1.7</td>
<td>47.3±0.9†</td>
<td>36.5±1.0‡</td>
</tr>
<tr>
<td>Fully muscular, %</td>
<td>0.3±0.3</td>
<td>2.0±0.7</td>
<td>6.3±1.4†</td>
<td>4.0±0.7</td>
</tr>
<tr>
<td>VWT of PAs (mm)</td>
<td>0.186±0.006 (4)</td>
<td>0.201±0.006 (4)</td>
<td>0.245±0.007† (4)</td>
<td>0.219±0.006‡ (4)</td>
</tr>
</tbody>
</table>

*Values are mean ± SE. Number of mice is indicated in parentheses.
†p < 0.05 vs. normoxia.
‡p < 0.05 vs. wild type + hypoxia.

**Definition of abbreviations:** Keap1 = Kelch-like ECH associating protein 1; Keap1f/f = Keap1 knockdown mice; PAs = pulmonary arteries; RV/LV + S = ratio of the right ventricular free wall to the combined weight of the left ventricle + septum; RVSP = right ventricular systolic pressure; VWT = vessel wall thickness (external diameter of PA – internal diameter)/external diameter.

This table presents the effects of Keap1 knockdown on hypoxia-induced pulmonary hypertension, right ventricular hypertrophy, muscularization of small pulmonary arterioles, and vessel wall thickness of the pulmonary arteries after 3 weeks of exposure to hypoxia. The table compares the effects in normoxic and hypoxic conditions, with and without Keap1 knockdown. The data show significant differences in various parameters between normoxic and hypoxic conditions, and between wild-type and Keap1 knockdown mice.
treatment in a dose-dependent manner. The inducible expression of these genes was barely detectable in Nrf2−/− mice administered oltipraz, indicating that oltipraz activates the expression of the antioxidant genes by inducing nuclear accumulation of Nrf2 protein in the mouse lung.

Chronic oltipraz administration attenuated hypoxia-induced cardiopulmonary alterations, as evidenced by the decreased RV/(LV + S) ratio, improved muscularization of small pulmonary arterioles, and suppressed PA wall thickening. Despite the remarkable improvement in the RVH and pulmonary vascular remodeling after oltipraz treatment in WT mice, the therapeutic efficacy of oltipraz was completely lost in Nrf2−/− mice. Therefore, the pharmacological activity of oltipraz against chronic hypoxia–induced cardiopulmonary alterations critically depends on Nrf2 function.

The preventive function of Nrf2 against chronic hypoxia–induced pulmonary vascular remodeling was further confirmed in the Keap1f/f mice subjected to the same 3-week hypoxic exposure. These mice exhibited marked resistance to chronic hypoxia–induced pulmonary vascular remodeling and RVH, thus indicating an essential Nrf2 function for the prevention of cardiopulmonary alterations in a chronic hypoxia–induced PH model. RVH was substantially exaggerated in Nrf2−/− mice compared with WT control mice. The dilation and hypertrophy of RV in hypoxia-exposed Nrf2−/− mice was macroscopically evident. However, hypoxia-exposed Nrf2−/− mice showed significantly less of an increased RVSP than hypoxic WT mice. Further experiments are required to determine whether this is related to a direct effect of Nrf2 on RV structure and function or whether this simply reflects a failing RV in which the RV can no longer generate an elevated RVSP in the face of the severe vascular remodeling. Other explanations are plausible, and this remains a fertile and important area for future investigation.

A correlation between RVSP and the development of RVH, as assessed by RV/(LV + S), has been reported in a hypoxic PH mouse model (23). However, both types of Nrf2 activation (i.e., oltipraz administration and Keap1 knockout) hardly exerted any positive effects on improvement of the increased RVSP level, whereas both showed an inhibitory effect against hypoxia-induced RVH and pulmonary vascular remodeling. Other explanations are plausible, and this remains a fertile and important area for future investigation.
but failed to decrease the development of hypoxic PH in rats (37). Because Rho-kinase signaling emerged as a major contribu-
tor to HPV pathogenesis, they administered a Rho-kinase in-
hibitor to hypoxic PH rats and successfully reduced the high PA
pressure to almost normal levels. Given this, Crossno and col-
leagues concluded that ROSI failed to block hypoxia-induced
PH primarily because of its inability to repress the sustained
Rho-kinase–mediated HPV. PPARγ and Nrf2 functionally
interact to synergistically elicit the protection against oxidative
lung injury (38). PPARγ expression level is up-regulated by
Nrf2, and PPARγ in turn activates Nrf2 expression (39). Thus,
ROSI would likely induce Nrf2 activity, whereas ROSI failed to
decrease the high PA pressure in the hypoxic rats. Taking these
findings into account, we surmise that the predominant con-
tribution of Rho-kinase–mediated HPV could not be attenuated
by Nrf2 activation or ROSI treatment. Therefore, PH could
remain, although hypoxia-induced pulmonary vascular remodel-
ing was efficiently decreased by oltipraz treatment or Keap1
knockdown.

Another potent Nrf2 activator, CDDO-imidazolide, has been
shown to attenuate right heart dysfunction in a murine model of
cigarette smoke–induced emphysema (40). Nrf2 activity pro-
tected against left cardiac hypertrophy, myocardial fibrosis
and apoptosis, heart failure, and increased mortality in a murine
heart failure model established by transverse aortic constric-
tion (41). These findings suggested that Nrf2 might exert a direct
cytoprotective function in cardiomyocytes under mechanical
stresses such as excessive afterload. In the present study, our
data also indicate a possible direct effect of Nrf2 on the RV, but
we did not control for differences in RV afterload, which were
likely present in Nrf2−/− mice. Isolated cardiac physiology experi-
ments in a Langendorf model (42) would be helpful to answer
these questions directly.

Hypoxic exposure has been shown to cause increased oxida-
tive stress in whole lung tissue in vivo (7, 8) and in cultured
bovine pulmonary endothelial cells in vitro (43). In this study,
we directly confirmed ROS accumulation in the CD31+ PVECs
of hypoxia-exposed Nrf2−/− and Nrf2+/+ mice by flow cytom-
etry analyses using DCFDA. Expectedly, 2 days of hypoxia
increased DCFDA levels in CD31+ cells in Nrf2−/− and
Nrf2+/+ lungs to further delve into these observations,
we examined the mRNA expression levels of Nrf2 and Nrf2
downstream antioxidant genes in a CD31+ cell fraction from
the lungs of hypoxic Nrf2−/− and Nrf2+/+ mice. Unexpectedly,
Nrf2 and its target genes were differentially expressed in
CD31+ cells on exposure to hypoxia. GCLC and HO-1 mRNA
expression in Nrf2−/− mice was down-regulated by hypoxic ex-
posure despite ROS accumulation. There is accumulating evi-
dence that the lungs of patients with respiratory disease and
hypoxemia show increased oxidative stress. However, alveolar
macrophages in patients with pulmonary emphysema exhibit
decreased expression of the Nrf2 target genes (44). Although
the mechanism underlying this observation is unknown, the
down-regulation of antioxidants in those patients likely contrib-
utes to the disease progression. Therefore, forced induction of
the antioxidant gene upon pharmacological induction of Nrf2
would elicit therapeutic efficacy to hypoxemic lung disease.

After exposure to hypoxia for 2 days, the NQO1 and GCLC
immunoreactivity became barely detectable in the PA wall of
WT mice. These data were consistent with the decreased expres-
sion of GCLC mRNA in the CD31+ cells from hypoxia-exposed
WT mice. However, the hypoxia-induced decrease in NQO1
expression in the whole lung was inconsistent with the constant
expression in the CD31+endothelial fraction. This ob-
ervation might be explained by the differential distribution of
GCLC and NQO1 in PA; NQO1 was mainly expressed in the PA

**Figure 4.** Effects of oltipraz treatment on the hypoxia-induced in-
tense Nrf2 (TN-C) expression in the lungs of Nrf2−/− mice. (A) qRT-PCR revealed that TN-C mRNA expression in the lungs of Nrf2−/− mice was significantly increased by 7 days of hyp-
oxia exposure (n = 4 each; normoxia + vehicle, 1.00 ± 0.11 versus hypoxia + vehicle, 2.55 ± 0.18; ∗P < 0.05). Oltipraz treatment (50 mg/kg/d for the entire 7 d of hypoxic exposure) significantly at-
tenuated the increase in lung TN-C mRNA levels caused by hypoxia
(n = 4 each; hypoxia + oltipraz, 1.93 ± 0.11; †P < 0.05 versus
hypoxia + vehicle levels). (B–H) Representative TN-C expression
pattern (brown staining) detected by immunohistochemistry (ori-
ignal magnification: ×400; bar represents 50 μm). AD = alveolar
duct; TB = terminal bronchiol. (B) Normoxic lung of a Nrf2−/−
mouse demonstrating faint staining for TN-C in bronchial epithelial
cells and in the external elastic lamina of the pulmonary artery wall.
(C) Nrf2−/− mice exposed to hypoxia for 7 days without oltipraz
administration showed strong Nrf2 expression pattern in the external
elastic lamina of the pulmonary artery wall. (D) Hypoxic Nrf2−/− mice
simultaneously treated with oltipraz showed decreased immunore-
activity to Nrf2 in the pulmonary artery wall compared with the
vehicle-treated hypoxic mice (C). (E–H) Close examination of lung
sections from vehicle-treated hypoxic Nrf2−/− mice revealed brown
signals for TN-C in the pulmonary artery walls corresponding to the
level of the terminal bronchiol (TB) (E, F, and arrows in G); no
stains were seen at the level of the alveolar duct (AD) (G and H,
arrowheads).
media, whereas GCLC expression was preferentially observed in the endothelium. Reduced NQO1 expression in the whole lung sample might be attributed to reduction of NQO1 in the PA media. Further analyses are necessary to evaluate antioxidant response in PA media under chronic hypoxia.

Another potential answer for the discrepancy may lie in the differences in the time-dependent changes of each Nrf2 target gene expression after hypoxia exposure. Several studies have demonstrated that the expression of the set of Nrf2 target genes was not always changed synchronously at the same time point after a single stimulus. For example, in the murine model of bleomycin-induced pulmonary fibrosis, the lung expression of GCLC mRNA, but not NQO1 and HO-1 mRNA, was significantly increased at 3 days after bleomycin injection (21). Meanwhile, the expression levels of NQO1 and HO-1 mRNA significantly increased at 7 days after bleomycin injection, whereas GCLC expression tended to decline at this time point. Another study using a neonatal murine model of hypoxic lung injury demonstrated that the expression of several Nrf2 target genes in lung tissue was differentially altered for 3 days after the initiation of 100% O2 exposure (45).

Our experimental design did not address the reason why some Nrf2 target genes were down-regulated despite an increase in ROS accumulation under hypoxic conditions. However, we surmised that hypoxia-inducible factor (HIF)α could suppress the Nrf2 activity in the hypoxia-exposed lung. Mechanistic insight into functional crosstalk between Nrf2 and HIFα systems has been largely obscured. It was reported that Nrf2-directed IL-8 and HO-1 expression was decreased by induction of HIFα activity (46). Another group also demonstrated that tertiary butylhydroquinone–induced HO-1 expression was decreased by hypoxia (47). They suggested that the increased HIF1α–MafG interaction under hypoxia might deprive the dimerization partner from Nrf2, thereby suppressing Nrf2 activity. Further functional validation is needed to detail the exact mechanism.

TN-C exerts antiapoptotic and growth-stimulatory effect on vascular smooth muscle cells and is up-regulated in lung tissues of pulmonary hypertensive animals (19, 20, 48). In a rat model of monocrotaline-induced PH, TN-C expression in the pulmonary vasculature is up-regulated by oxidative stress (48). In bleomycin-induced lung fibrosis, TN-C is more abundantly accumulated in Nrf2−/− mice than in Nrf2+/− mice (21). Thus, Nrf2 activity presumably plays an important role in preventing aberrant TN-C accumulation in diseased lung. TN-C mRNA abundance was significantly increased after 1 week of hypoxia exposure, whereas the TN-C mRNA level was significantly decreased by oltipraz treatment. Immunohistochemical analyses with TN-C antibody demonstrated that the increased TN-C signal was localized in the external elastic lamina of the PA walls, especially at the level of the TB in hypoxic mice. Moreover, Keap1f/f mice showed significantly less TN-C signals in PA walls compared with hypoxic Nrf2−/− mice (Figures E4G, E4I, E4J, and E4K). The level of TN-C signals in the PA walls of hypoxic Keap1f/f mice was comparable with that of nontreated hypoxic Nrf2−/− mice (Figures E4G, E4H, E4I, and E4K). Comparable TN-C staining in lung tissues of hypoxia-exposed Nrf2−/− and Nrf2+/− mice may account for the similar extent of hypoxia-induced pulmonary vascular remodeling in these mice. decreased TN-C immunoreactivity in the PA walls of oltipraz-treated hypoxic Nrf2−/− mice and hypoxia-exposed Keap1f/f mice suggested that oltipraz or Keap1 knockdown decreases hypoxia-induced pulmonary vascular remodeling in part through inhibition of lung TN-C accumulation.

In our patient with COPD associated with PH, the expression of GCLC and NQO1 was diminished in the endothelial layers of PA walls, in good agreement with the decreased GCLC expression in CD31+ PVECs in hypoxia-exposed WT mice. However, IPAH lung showed strong GCLC and NQO1 signals in pleomorphic lesions and endothelial layers of thickened PA, presumably responding to oxidative stress (27). The differential response to ROS accumulation between COPD+PH and IPAH would provide attractive subjects for future studies, which would require another model of IPAH.

The present study showed for the first time that pharmacological and genetic activation of Nrf2 was capable of decreasing hypoxia-induced RVH and pulmonary vascular remodeling in a PH animal model. Nrf2 activators may prove to be a useful adjunct to currently available therapies for PH associated with respiratory diseases presenting with hypoxemia, such as COPD and IPF.

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgments: The authors thank Dr. Yoshihiro Fukumoto, Department of Cardiovascular Medicine, Tohoku University Graduate School of Medicine; Dr. Tsutomu Tamada, Department of Respiratory Medicine, Tohoku University Graduate School of Medicine; Dr. Shozaburo Doi, Department of Pediatrics and Developmental Biology, Tokyo Medical and Dental University; and Dr. Masayuki Noda, Department of Thoracic Surgery, Institute of Development, Aging and Cancer, Tohoku University for helpful discussions and Masakazu Honda, Department of Thoracic Surgery, Institute of Development, Aging and Cancer, Tohoku University for technical assistance.

References


