Adrenomedullin Can Protect Against Pulmonary Vascular Remodeling Induced by Hypoxia

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Background—Chronic hypoxia is one of the major causes of pulmonary vascular remodeling associated with stimulating reactive oxygen species (ROS) production. Recent studies have indicated that hypoxia upregulates expression of adrenomedullin (AM), which is not only a potent vasodilator but also an antioxidant. Thus, using heterozygous AM-knockout (AM+/−) mice, we examined whether AM could attenuate pulmonary vascular damage induced by hypoxia.

Methods and Results—Ten-week-old male wild-type (AM+/+) or AM+/− mice were housed under 10% oxygen conditions for 3 to 21 days. In AM+/+ mice, hypoxia enhanced AM mRNA expression, which was reduced by the administration of a superoxide dismutase mimetic, 4-hydroxy-2,2,6,6-tetramethyl-piperidine-N-oxyl (hydroxy-TEMPO). Hypoxia induced pulmonary vascular remodeling, which was associated with the increased production of oxidative stress measured by electron spin resonance and immunostaining of 3-nitrotyrosine. The media wall thickness of the pulmonary arteries was significantly greater in AM+/− mice housed under hypoxia than in AM+/+ mice under hypoxia. Concomitantly, pulmonary ROS production induced by hypoxia was more enhanced in AM+/− mice than in AM+/+ mice. The administration of both exogenous AM and hydroxy-TEMPO normalized pulmonary vascular media wall thickness in not only AM+/+ but also AM+/− mice under hypoxic conditions associated with the normalization of ROS overproduction in the lung.

Conclusions—The present results suggest that an endogenous AM is a potential protective peptide against hypoxia-induced vascular remodeling, possibly through the suppression of ROS generation, which might provide an effective therapeutic strategy. (Circulation. 2004;109:2246-2251.)

Key Words: mice, knockout remodeling stress, oxidative hypertension, pulmonary

Chronic hypoxia is one of the major pathophysiological causes of pulmonary vascular remodeling. Long-term hypoxic animal models are known to exhibit the overproduction of reactive oxygen species (ROS). An excess of ROS has been suggested to trigger pulmonary arterial vasoconstriction through the increment of cytosolic calcium in the pulmonary arterial myocyte, which appears to operate as an autoregulatory mechanism to limit blood flow to hypoxic alveoli and to preserve ventilation-perfusion matching.

However, the overproduction of ROS results in the progression of pulmonary vascular remodeling. ROS upregulates expression of several factors to modify vascular remodeling, such as vascular endothelial growth factor (VEGF), platelet-activating factor (PAF), and mitogen-activated protein kinase (MAPK). Thus, several intrinsic substances to modulate pulmonary ROS levels may play protecting or aggravating roles in progression of pulmonary vascular remodeling.

In our previous in vitro study, hypoxia induced higher expression of a potent vasodilator peptide, adrenomedullin (AM), in vascular endothelial cells. In addition, AM and its receptor expression were enhanced in the lung of chronic hypoxic animal models. Also, in the clinical setting, plasma AM concentration was increased in patients with pulmonary hypertension associated with mitral stenosis. AM could have a potential organ-protective action, probably by reducing oxidative stress. In fact, AM inhibited ROS generation in mesangial cells, which may be related to the reduction of MAPK-mediated mitogenesis. In support of this, our previous report indicated that AM-deficient mice with angiotensin II and salt loading showed severe coronary damage associated with ROS excess. Because AM expression is upregulated by ROS itself, as we previously reported, ROS-induced endogenous AM overproduction might act as one of the feedback mechanisms to inhibit hypoxia-induced overproduction of ROS.

In the present study, we investigated the involvement of AM and ROS in protection against hypoxia-induced pulmonary vascular remodeling.
**Methods**

**Animals and Hypoxic Conditions**

AM-null mice were generated as described previously. In brief, a stop mutation was inserted at the starting point of the AM coding sequence on exon 4, by which AM peptide was disrupted despite normal production of proadrenomedullin N-terminal 20 peptide, an alternative product of the AM gene, to decrease blood pressure by sympathoinhibition. Because complete AM deficiency is lethal to mouse embryos, we used heterozygous AM-deficient (AM+/−) mice. We have previously demonstrated that in AM−/− mice, AM content was approximately half that of the wild-type (AM+/+) mice in plasma, adrenal glands, and other organs. Moreover, AM overexpression mechanisms were not operating sufficiently under several AM-stimulating conditions in AM+/− mice and AM−/− littermates were housed under room air or 10% oxygen conditions in a normobaric chamber for 3 to 21 days. The hypoxic environment was maintained by continuous mixed gas flow (10% oxygen/90% nitrogen gas, 300 mL/min). The chamber was opened for 5 minutes once every 2 days for feeding and changing animal cages. After the chamber was closed, the mixed gas was flushed to recover the hypoxic environment as quickly as possible. Animals were maintained at 26±2°C with a 12:12-hour light-dark cycle. All animals were fed standard mouse chow and water ad libitum. Mice were handled in an accredited institute facility in accordance with the institutional animal care policies, and all research protocols conformed to the guiding principles for animal experimentation as articulated by the Ethics Committee on Animal Research of the University of Tokyo.

**AM Supplementation and Hydroxy-TEMPO Administration**

Exogenous AM (30 ng · kg−1 · h−1) was injected into the peritoneal cavity of the AM+/− mice or AM−/− mice with a micro-osmotic pump, Alzet 1002 (Durect Co), during hypoxic exposure. N-acetyl-L-cysteine (NAC), a superoxide dismutase mimetic, was administered in the drinking water (10 mmol/L). Hydroxy-TEMPO is detectable by ESR spectra but rapidly traps electrons from the hydroxy radical to change them into hydroxylamine compounds, which is undetectable. Thus, the rate of signal decay of hydroxy-TEMPO is proportional to the ROS amount. We recorded ESR spectra every 2 minutes and calculated the rate of signal decay to quantify the amount of ROS.

**Evaluation of Change in Pulmonary AM Expression Under Hypoxia in AM+/− Mice**

After 3 and 7 days of hypoxic exposure or 7 days of room air, mice were euthanized with pentobarbital (1g/kg body wt IP), and lungs were excised bilaterally. The mRNA was extracted by the guanidium method (ISOGEN, Nippon Gene), and semiquantitative polymerase chain reaction (PCR) was performed (denaturation at 95°C for 1 minute, annealing at 55°C for 1 minute, extension at 72°C for 1 minute). Sense or antisense primers were designed to locate the site opposite each other crossing between the intron coding region, and the length of the production was 173 bp (sense primer: 5’-TCAGAGCATCGCCACAGAAT-3; antisense primer: 5’-TAGCTGCTGATGCTTGAG-3’). The amount of mRNA production was semiquantified by measuring fluorescence density of the band of 173 bp and standardized with ribosomal RNA, 18S product, using Image Scan Soft ware (Scion Image).

**Quantification of Damage of Pulmonary Artery**

Lung tissues after 21 days of hypoxic exposure were sampled after injection of 4% paraformaldehyde from trachea to expand the alveoli. Lungs were fixed with 4% paraformaldehyde, embedded in paraffin, and subsequently cut into sections 3 μm thick and were stained with hematoxylin and eosin. The morphological changes of the small pulmonary arteries (external diameter: 80 to 120 μm) were estimated by media wall thickness (%MT), which was calculated by (media thickness−2 × external diameter) × 100.

The width of the media and external diameter were measured using Image Scan Software (Scion Image). %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 not being perfectly symmetrical circles.

**Evaluation of Reactive Oxygen Species**

To quantify ROS generation at the lung in real time, we used the electron spin resonance (ESR) method as reported previously. After 3, 7, and 21 days of hypoxic exposure, mice were anesthetized with pentobarbital, and PBS was injected into the right ventricle with a 26-gauge needle to wash blood away from the pulmonary vascular bed, by which we could minimize the influence of ROS generated from white blood cells. After that, excised left lung tissue was homogenized in PBS liquid containing protease inhibitor, and the tissue was reacted immediately with a low-molecular-weight spin probe, 0.1 mmol hydroxy-TEMPO. The details of the ESR setting were as follows: a microwave power of 10 mW, a range of external magnetic field of 10 mT, and a scan rate of 1 mT/s, and measurements were performed at room temperature with an ESR spectrometer (JES-FA-100; JEOL). Hydroxy-TEMPO is detectable by ESR spectra but rapidly traps electrons from the hydroxy radical to change them into hydroxylamine compounds, which is undetectable. Thus, the rate of signal decay of hydroxy-TEMPO is proportional to the ROS amount. We recorded ESR spectra every 2 minutes and calculated the rate of signal decay to quantify the amount of ROS.

To localize ROS production, immunohistochemistry of 3-nitrotyrosine was performed. After paraffin was removed from prepared lung tissue, sections were rehydrated, and slides were treated with 10 mmol/L citric acid (pH 6.0) and boiled 3 times by microwaves. Non specific binding was blocked with 10% normal goat serum (pH 7.4) for 30 minutes. Subsequently, samples were incubated with 5 μg/mL anti-3-nitrotyrosine antibody (Upstate Biotecnology) with 1% BSA for 1 hour at 37°C and then incubated with a biotinylated anti-rabbit IgG secondary antibody with the Vectastain ABC kit (Vector Laboratories). Diaminobenzidine substrate was used to visualize positive immunoreactivity.

**Statistical Analysis**

All values were expressed as mean±SEM. Comparisons among groups were made by ANOVA followed by Schéffe’s method, and probability values of P<0.05 were considered to indicate statistical significance.

**Results**

**Hypoxic Change of AM Expression**

In AM+/− mice, pulmonary AM expression was enhanced during chronic hypoxia, which was normalized to the same as that of normoxic mice with the administration of hydroxy-TEMPO (Figure 1, a and b). AM expression was significantly enhanced after 3 days of hypoxic exposure; it gradually decreased after 7 days, the level of which was slightly but not significantly higher than that of normoxic mice. In contrast, in AM−/− mice, AM expression remained unchanged during chronic hypoxia (data not shown).

**Hypoxic Change of Pulmonary Vessel Morphology and ROS Generation**

Hypoxia caused moderate pulmonary vascular remodeling in AM+/− mice, quantified by %MT (normoxia, 41.2±1.8% [Figure 2a]; hypoxia, 49.3±2.7% [Figure 2b]; P<0.05) (Figure 3). Conversely, hypoxia induced severe pulmonary vascular damage in AM−/− mice (normoxia, 42.9±6.0% [Figure 2e]; hypoxia, 66.6±3.4% [Figure 2f]; P<0.01) (Figure 3): %MT was greater in the AM−/− mice under hypoxic conditions than in the hypoxic AM−/− mice (P<0.01), al-
though %MT was not different between AM/H1/H1 and AM/H1/H1 mice under normoxic conditions (Figure 3). Hypoxia elevated pulmonary ROS production in both AM/H1/H1 and AM/H1/H1 mice, but the degree of hypoxia-induced ROS generation was significantly greater in AM/H1/H1 mice, which was shown by both ESR signal decay (Figure 4) and pulmonary arterial 3-nitrotyrosine immunostaining (Figure 5, a and d). The localization of 3-nitrotyrosine staining coincided with endothelial cells of the pulmonary artery. In addition, the time course of ROS overproduction with chronic hypoxia was similar to AM expression in AM/H1/H1 mice (Figures 1 and 4); ROS production and AM expression reached their peak 3 days after hypoxic exposure and thereafter decreased gradually. Note that the levels of ROS production on 3 and 7 days after hypoxia in AM/H1/H1 mice were significantly higher (P<0.05 and P<0.01) than those in hypoxic AM/H1/H1 mice (Figure 4).

Effects of Exogenous AM Supplementation and Hydroxy-TEMPO Administration to the Mice Under Hypoxia
To elucidate the possible role of AM in pulmonary vascular damage, we evaluated the effect of exogenous AM supplementation on the pulmonary vasculature and ROS generation. AM normalized %MT in both AM/H1/H1 and AM/H1/H1 mice treated with hypoxia to the same level as mice with normoxia; P<0.05, P<0.01, respectively) (Figure 2, c and g; Figure 3); This level was not different from that of the AM/H1/H1 mice with normoxia. The reduction of %MT was accompanied by decreased ROS production measured by ESR (Figure 4) and pulmonary immunostaining of anti–3-nitrotyrosine (Figure 5, b and e). In addition, to clarify the role of ROS in the development of pulmonary arterial remodeling, we treated with a superoxide dismutase mimetic, hydroxy-TEMPO. Hydroxy-TEMPO reduced ROS production (Figure 4 and Figure 5, c and f). Concordantly with the reduction of ROS, in both AM/H1/H1 and AM/H1/H1 mice, %MT was also improved to the same level as in mice with normoxia; P<0.01, respectively) (Figure 2, d and h, and Figure 3).

Discussion
In the present study, we showed that chronic hypoxia induced pulmonary vascular damage associated with ROS overproduction in the lung. In hypoxic conditions, ROS generation
was intimately related to AM expression, because the time course of AM expression in the lung from AM−/− mice was similar to that of ROS generation quantified by ESR; both the AM expression and ROS generation were moderately increased during the early period of hypoxia. In addition, the treatment of hydroxy-TEMPO, a membrane-permeable superoxide dismutase mimetic, could normalize the hypoxia-induced overexpression of AM. Moreover, in AM−/− mice, chronic hypoxia resulted in more severe vascular damage associated with greater ROS production compared with AM+/+ mice. Because hypoxia-induced medial hyperplasia of pulmonary arteries was completely reversed by either AM or hydroxy-TEMPO treatment, AM might be one of the important intrinsic compensatory substances to protect against hypoxia-induced pulmonary vascular remodeling, possibly through the suppression of ROS generation.

AM can be recognized as a potent antioxidative and antiatherosclerotic substance. In support of this, we previously revealed that the lack of AM resulted in acceleration of vascular damage in coronary arteries in an angiotensin II− and salt-loaded model associated with increased ROS production. We have demonstrated organ-protective effects of AM through its antioxidant action not only in coronary arterial damage induced by angiotensin II and salt loading but also in pulmonary vascular remodeling induced by hypoxia. The protective effect of AM may be generalized to several kinds of organ damage caused by ROS generation. In fact, we have demonstrated that intrinsic AM prevented age-related progression of insulin resistance via suppression of ROS overproduction. Thus, AM is one of the important intrinsic

Figure 3. Quantification of small pulmonary artery remodeling by %MT after 21 days of hypoxia (n=3 to 4 for each group). Hypoxia induced media hyperplasia, which was more severe in AM−/− mice than in AM+/+ mice (P<0.01). AM sup indicates AM supplementation. *P<0.05 vs normoxic AM+/+ mice. †P<0.05, ††P<0.01 vs hypoxic AM+/+ mice without treatment. ‡P<0.01 vs normoxic AM−/− mice. §§P<0.01 vs hypoxic AM+/− mice without treatment.

Figure 4. ROS measured by ESR analysis of hydroxy-TEMPO in lung (n=3 to 4 for each group). AM sup indicates AM supplementation. *P<0.05 vs normoxic AM+/+. †P<0.05, ††P<0.01 vs hypoxic AM+/+ mice without treatment. ‡P<0.05 vs normoxic AM−/− mice. §§P<0.05, §§§P<0.01 vs hypoxic AM+/− mice without treatment.

Figure 5. Representative photomicrographs of immunostaining by 3-nitrotyrosine of pulmonary arteries of mice housed under hypoxic conditions. a, Hypoxic AM+/+ mice, b and c, Hypoxic AM+/− mice treated with AM (b) and hydroxy-TEMPO (c), d, Hypoxic AM−/− mice, e and f, Hypoxic AM+/− mice treated with AM (e) and hydroxy-TEMPO (f). Red arrows indicate pulmonary arterial endothelial cells.
factors to protect organs against the overproduction of oxidative stress.

The precise mechanisms of ROS inhibition by AM have not been revealed; however, it has been reported that AM suppresses ROS production in a dose-dependent manner via activation of the cAMP–protein kinase A pathway in mesangial cells. In a rat myocardial ischemia–reperfusion injury model, gene delivery of AM suppressed ROS production by NADPH oxidase inhibition via the nitric oxide (NO)–cGMP signaling pathway. In addition, because AM induced vasodilation because of increased cAMP and NO overproduction and endothelin-1 downregulation, AM-induced reduction of shear stress might be involved in reducing ROS production. But we performed no hemodynamic studies.

In the present study, treatment with hydroxy-TEMPO attenuated ROS production and reversed pulmonary vascular remodeling. Evidence is accumulating that ROS is the upstream signal of chronic hypoxia–induced pulmonary vasoconstriction and development of vascular remodeling. It has been reported that the ROS generated by hypoxia can induce calcium release from sarcoplasmic reticulum stores, followed by pulmonary vasoconstriction. Moreover, ROS activated VEGF, PAF, and MAPK, which induced vascular remodeling. PAF subsequently induced oxidative bursts in macrophages, which might cause a vicious circle to advance the process of pulmonary vascular remodeling. In the early phase of hypoxic conditions, ROS may act as a trigger of this signal cascade in the process of vascular remodeling, because, in the present study, the ROS level quantified by ESR was transiently elevated at 3 days of hypoxia and subsequently decreased in AM−/− mice; the ROS level at 7 to 21 days of hypoxia was not significantly different from that of normoxia. This finding is consistent with the previous results in rats that the ROS level was increased only during the early period of hypoxic exposure. This finding led us to speculate that ROS might be the cause but not the result of hypoxia-induced pulmonary vascular damage. The precise mechanism of the reduction of ROS at the late phase has not been elucidated; however, there may be some feedback system, such as the activation of superoxide dismutase or glutathione peroxidase.

Alternatively, AM might be one of the candidates for the ROS regulator, because the increased AM in AM−/− mice, in turn, could reduce ROS production. In contrast, the failure of ROS-induced AM increase in AM−/− mice caused the sustained increment of ROS generation. Taken together, it might be a plausible concept of treatment that strategies for ROS reduction during the early period of hypoxia may be efficacious for the therapy of hypoxic vascular remodeling.

In this regard, AM has advantages in treating pulmonary vascular remodeling because of its ROS-inhibitory effect in addition to the hemodynamic improvement by pulmonary vasodilation. We did not evaluate pulmonary hemodynamic changes induced by AM because we were technically limited to keep mice under hypoxia during examination. However, it has been reported that active treatment with AM infusion effectively reduced pulmonary arterial pressure and attenuated pulmonary arterial resistance in a small number of patients with primary pulmonary hypertension. Although problems still remain to be solved, such as the optimal dose of AM and the way to administer AM, gene delivery might be one possible way. Our previous study indicated that gene delivery of AM could inhibit cuff-induced arterial intimal hyperplasia in rats. Cardiac remodeling and renal injury were attenuated by gene delivery of AM in deoxycorticosterone acetate–salt hypertensive rats. As for treatment of pulmonary vascular damage, intratracheal gene delivery can be an effective method, which was shown by the study of adenoviral vector administration encoding calcitonin gene–related peptide in hypoxic mice. Transplantation of AM gene–transduced endothelial progenitor cells was reported to ameliorate monocrotaline-induced pulmonary hypertension in rats. Taken together, AM might give a new clue to the therapeutic strategy against pulmonary vascular remodeling via ROS suppression.

In summary, hypoxic AM−/− mice showed not only severe pulmonary vascular injury but also higher levels of ROS production, both of which were reduced by AM supplementation as well as the treatment with hydroxy-TEMPO. AM has a protective effect against hypoxia-induced pulmonary vascular remodeling, possibly through the inhibition of ROS production.

References


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