L-Arginine restores endothelial nitric oxide synthase-coupled activity and attenuates monocrotaline-induced pulmonary artery hypertension in rats

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Ou ZJ, Wei W, Huang DD, Luo W, Luo D, Wang ZP, Zhang X, Ou JS. L-Arginine restores endothelial nitric oxide synthase-coupled activity and attenuates monocrotaline-induced pulmonary artery hypertension in rats. Am J Physiol Endocrinol Metab 298: E1131–E1139, 2010. First published March 9, 2010; doi:10.1152/ajpendo.00107.2010.—L-Arginine can attenuate pulmonary hypertension (PH) by a mechanism that are not fully understood. This study investigated the molecule mechanism of 1-arginine attenuating PH. Sprague Dawley rats were treated with monocrotaline (MCT) with or without l-arginine for 3 or 5 wk. Right ventricular systolic pressure (RVSP), right heart hypertrophy, survival rate, pulmonary artery wall thickness, nitric oxide (NO) concentration, and superoxide anion (O2·-) generation in the lung were measured. Expressions of endothelial nitric oxide synthase (eNOS) and heat shock protein 90 (HSP90) and heat shock protein 90 (HSP90) were measured. MCT increased RVSP, right heart hypertrophy, mortality, pulmonary artery wall thickness, and O2·- generation and decreased eNOS and HSP90 expression and association, phosphorylation of eNOS at Ser1177, and NO production. L-Arginine decreased RVSP, right heart hypertrophy, mortality, O2·- generation, and pulmonary artery wall thickness and increased NO production. L-Arginine increased eNOS expression, phosphorylation of eNOS at Ser1177, and association of eNOS and HSP90 without significantly altering HSP90 expression. L-Arginine may act through three pathways, providing a substrate for NO generation, preserving eNOS expression/phosphorylation, and maintaining the association of eNOS and HSP90, which allows restoration of eNOS activity and coupling activity, to maintain the balance between NO and O2·- and delay the development of PH.

mean pulmonary arterial pressure, pulmonary vascular resistance, and PH mortality in patients and in animal models (4, 5, 8, 26–29, 38, 46). However, the molecular mechanisms by which l-Arg attenuates PH remain unclear.

Previous studies conducted in different animal models demonstrated that l-Arg reduces generation of superoxide anion (O2·-) and prevents the reduced expression of eNOS (40, 46). The balance between NO and O2·- plays an important role in the process of many cardiovascular and pulmonary diseases (43). We and others (9, 31, 35) have shown that the interaction between eNOS and heat shock protein 90 (HSP90) is crucial in regulating this balance. Studies using cultured endothelial cells isolated from PH reported decreased NO production and increased O2·- generation associated with dissociation of eNOS with HSP90 (1, 17). We also showed in a PH animal model that eNOS expression is reduced in pulmonary arteries and peripheral lung and that the association of eNOS with HSP90 is decreased in pulmonary artery and in hypertensive endothelial cells (18). Although the use of various animal models has demonstrated l-Arg-mediated increases in NO production and eNOS expression, and reduced O2·- generation (5, 8, 40, 46), whether l-Arg affects the interaction of eNOS with HSP90 and the NO and O2·- balance in PH remains unknown. This study was designed to explore the impact of l-Arg on such interaction and balance in an MCT-induced PH rat model.

MATERIALS AND METHODS

Establishment of PH model. All animal experiments were approved by The First Affiliated Hospital, Sun Yat-sen University Review Board and Animal Research Committee. The investigation conformed to the provisions of the Declaration of Helsinki in 1995 (as revised in Edinburgh, 2000). Male Sprague-Dawley rats were injected intraperitoneally (ip) with a single dose (50 mg/kg) of MCT (Sigma-Aldrich, St. Louis, MO) to induce PH as previously described (39). Control rats received an equal volume of isotonic saline.

Study groups. Six groups were studied. A control group (n = 20) received a daily ip injection of saline for 5 wk. Animals in the l-Arg group (n = 20) received an ip dose of 300 mg/kg l-Arg (Sigma-Aldrich) each day for 3 wk [this dose has been shown to inhibit the development of PH (5, 15, 28)]. The MCT3 group (n = 26) consisted of rats treated with a single dose of MCT and a daily ip injection of saline for 3 wk. The MCT3/l-arg3 group (n = 26) was comprised of MCT-treated rats also injected ip with 500 mg/kg l-Arg daily for 3 wk. The MCT5 group (n = 26) consisted of rats treated with a single dose of MCT and a daily ip injection of saline for 5 wk. Finally, the MCT5/l-arg5 group (n = 26) consisted of rats treated with a single dose of MCT who also received a daily ip injection of l-Arg (500 mg/kg) for 5 wk. Body weight was recorded at the end of the experimental period, except for control rats, whose weight was mea-

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sured at both 3 and 5 wk. Another 60 control, MCT, and MCT/-arg rats were used for survival comparison. At the conclusion of the experiments, the subsequently described determinations were carried out.

Surgical preparation and measurement of pulmonary arterial pressure. Pulmonary artery pressure was measured using methods described previously (24, 39). The animals were initially anesthetized with an ip injection of pentobarbital sodium. A right heart catheter (PE-50 tubing) was inserted through the right jugular vein to measure the right ventricular systolic pressure (RVSP) as an indicator of pulmonary artery pressure. After this measurement, the chest was opened, and the right hilus pulmonis was ligated. The right lung of each animal was excised and divided by the upper and lower lobes. Each upper lobe was fixed using 10% formaldehyde, embedded in paraffin, and sectioned (10 μm thickness). Sections were stained for hematoxylin and eosin (H&E) elastic staining. One-half of the lower lobes of each group were used for NO measurement, and the remaining half was frozen in liquid nitrogen for Western blot and immunoprecipitation studies. The left lung of each animal remained in the body and was perfused with hydroethidine to measure O2·− generation.

Effects of l-Arg on pulmonary O2·− generation. A low-flow syndrome pump with an attached 60-ml syringe was connected to the main pulmonary artery through the right ventricle. A tight ligature was placed around the main trunk of the pulmonary artery. The left lung was perfused with 10 μM hydroethidine (HE; Ana Spec, Fremont, CA) in MOPS buffer at 9 ml/min for 10 min. The rat was covered with aluminum foil during this period to avoid light. l-Nitroarginine methyl ester (l-NAME; 500 μM, Sigma-Aldrich) dissolved in MOPS buffer was perfused into the lung at a rate of 9 ml/min for 20 min before perfusion with H&E in one-half of the animals from groups MCT3 and MCT5. After perfusion, each left lung was excised, snap frozen, and sectioned (10 μm thickness). Fluorescence images of oxidized HE in the lung were recorded using fluorescence microscopy. The range of fluorescent intensities was assessed using NIH Image J software. O2·− generation was reported in terms of relative changes in pixel intensity in arbitrary units as described previously (31, 42).

Assessment of right ventricular hypertrophy. After perfusion, the heart of each rat was removed, and the atria were removed. The right ventricle (RV), septum (S), and left ventricle (LV) were separated. The ratio of RV to LV plus S weight (RV/LV + S) was calculated as an index of right ventricular hypertrophy as previously described (39).

Effects of l-Arg on NO generation in lung. After perfusion, the lower lobe of each right lung was removed and placed on 1:9 (wt/vol) MCT3 and MCT5. After perfusion, each left lung was excised, snap frozen, and sectioned (10 μm thickness). Fluorescence images of oxidized HE in the lung were recorded using fluorescence microscopy. The range of fluorescent intensities was assessed using NIH Image J software. O2·− generation was reported in terms of relative changes in pixel intensity in arbitrary units as described previously (31, 42).

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Effects of l-Arg on NO generation in lung. After perfusion, the lower lobe of each right lung was removed and placed on 1:9 (wt/vol) cold homogenized buffer. The lungs were cut into small pieces using an iris scissors and homogenized five times on ice (10 s with a 30-s intervals between homogenizations). The homogenates were then centrifuged at 2,000 rpm for 8 min at 4°C. The supernatant was placed around the main trunk of the pulmonary artery. The left lung of each animal remained in the body and was perfused with hydroethidine to measure O2·− generation.

Effects of l-Arg on pulmonary arterial vessel wall. Five-micrometer-thick sections were made from paraffin-embedded lung blocks. H&E elatica staining was performed, and the slides were examined by light microscopy in a blinded fashion without the knowledge of the treatment groups. In each section, 40–50 intra-acinar arteries were counted. Each artery was first landmarked by its accompanying airway (i.e., terminal bronchioles, respiratory bronchioles, alveolar duct, or wall), its smallest external diameter was measured, and the mean arterial size was then calculated. In addition, each artery was identified as being of one of three structural types: muscular (with a complete medial coat of muscle), partially muscular (with an incomplete coat, only a crescent of muscle being present), or nonmuscular (no muscle apparent), as previously described (37). The percentage of fully muscularized arteries was determined. Wall thickness was measured at the two ends of the shortest external diameter and the average was taken. The percent wall thickness was calculated as [diameter (%) = (2 × wall thickness + external diameter) × 100].

Western analysis and immunoprecipitation. The expression of eNOS and HSPP90, phosphorylation of eNOS at Ser1177, and HSPP90 interaction with eNOS in lung were determined as described previously (18, 19, 32). Briefly, frozen lung samples were pulverized and placed in a modified RIPA buffer (18). The extract was then homogenized and sonicated to break the cells, and the cell debris was removed by centrifugation at 14,000 g for 10 min at 4°C. The supernatant was transferred to a cold microcentrifuge tube, and protein concentrations were determined by a bicinchoninic acid protein assay. The protein was used for Western analysis and immunoprecipitation. eNOS was immunoprecipitated with 1 μg of H-32 antibody (BioMol, Plymouth Meeting, PA) per 100 μg of protein (500 μg total), as described (18, 19, 32). Antibodies for detecting eNOS (Santa Cruz Biotechnology, Santa Cruz, CA), HSPP90 (Santa Cruz Biotechnology), phosphorylation of eNOS at Ser1177 (Cell Signaling Technology, Danvers, MA), and β-actin (Biosynthesis Biotechnology, Beijing, China) were used. Bands were visualized using horseradish peroxidase (HRP)-linked secondary antibodies and enhanced chemiluminescence using ImmunoStar reagents (Santa Cruz Biotechnology). Images of the bands of interest in the autoradiograms were obtained with an EPSON scanner and Adobe Photoshop CS. Densities of the bands were quantified from the scanned images using NIH Image 1.63 software. Relative changes in eNOS phosphorylation (P-eNOS) at Ser1177 (M) hydroethidine (HE; Ana Spec, Fremont, CA) in MOPS buffer at 9 ml/min for 10 min at 4°C. The supernatant was transferred to a cold microcentrifuge tube for NO assay, and protein concentrations were determined by a bicinchoninic acid protein assay (Merck, Whitehouse Station, NJ). NO was determined spectrophotometrically by measuring total nitrate plus nitrite (NO3−) with a NO detection kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer’s instructions. Briefly, nitrate was enzymatically converted into nitrite by nitrate reductase, and nitrate was quantified using Griess reagent at an absorbance of 550 μM as previously described (21, 47). Results were expressed as micromoles per gram of protein.

Effects of l-Arg on pulmonary arterial vessel wall. Five-micrometer-thick sections were made from paraffin-embedded lung blocks. H&E elatica staining was performed, and the slides were examined by light microscopy in a blinded fashion without the knowledge of the treatment groups. In each section, 40–50 intra-acinar arteries were counted. Each artery was first landmarked by its accompanying airway (i.e., terminal bronchioles, respiratory bronchioles, alveolar duct, or wall), its smallest external diameter was measured, and the

RESULTS

l-Arg attenuates PH. As shown in Fig. 1A, MCT dynamics induced PH in rats. RVSP reached 31.05 ± 2.36 and 49.66 ± 3.283 mmHg at 3 and 5 wk post-MCT, respectively, compared with the control value of 11.75 ± 1.913 mmHg (P < 0.05, n = 20–26). l-Arg significantly attenuated MCT-induced PH, with values for RVSP being 21.14 ± 2.136 and 35.87 ± 2.437 mmHg at 3 and 5 wk post-MCT, respectively (P < 0.05, n = 20–26). l-Arg alone had no effect on pulmonary artery pressure by (10.37 ± 1.821 vs. 11.75 ± 1.913 mmHg, P > 0.05, n = 20). The right ventricular hypertrophy data confirmed
RVSP findings; MCT induced RV/LV increase and L-Arg diminished the MCT-induced RV/LV increase ($P < 0.05, n = 20–26; \text{Fig. 1B}$).

L-Arg prolongs survival in MCT-induced PH rats. During the experiment period, four animals died in group MCT5 and one died in group MCT5/L-arg. Thus, to determine the effect of L-Arg on the survival rate in MCT-induced PH, another set of experiments (60 rats, $n = 20$ in each group) were performed. Some animals began to die 4 wk after receiving an injection dose of MCT injection (95% survival at 4 wk, 60% survival at 6 wk). Interestingly, no animal deaths occurred in the L-Arg treatment group 4 wk post-MCT, and the survival rate at 6 wk was 85%. All rats of the control group survived for the entire experimental period ($P < 0.05, n = 20$ each; Fig. 1C). MCT-treated animals displayed decreased body weight that was less than that of control animals ($P < 0.05, n = 20–26; \text{Fig. 1D}$). L-Arg treatment prevented body weight loss in MCT-treated animals ($P < 0.05, n = 20$; Fig. 1D).

L-Arg restores NO generation in MCT-induced PH lung. NO concentration in the lung was significantly decreased at 3 and 5 wk post-MCT compared with control ($P < 0.05, n = 10$; Fig. 2). Although L-Arg itself slightly increased NO production, no statistical significance was found when compared with control animals. L-Arg partially restored NO generation both at 3 and 5 wk post-MCT ($P < 0.05, n = 10$; Fig. 2).

L-Arg inhibits $O_2^-$ generation in MCT-induced PH lung. To determine the effect of L-Arg on $O_2^-$ generation in MCT-induced PH in vivo, left lungs were perfused with H&E. Figure

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**Fig. 1.** Post-monocrotaline (MCT) observations. A: right ventricular systolic pressure (RVSP) was markedly elevated at 3 and 5 wk post-MCT. There was a significant reduction in RVSP in rats receiving L-arginine (L-Arg). *MCT3, MCT5 vs. Control; #MCT3/L-arg3 vs. MCT3; &MCT5/L-arg5 vs. MCT5; $P < 0.05$. B: there was a significant increase in RV:LV ratio at 3 and 5 wk post-MCT. L-Arg inhibited RV:LV ratio increase in MCT-treated animals. *MCT3, MCT5 vs. Control; #MCT3/L-arg3 vs. MCT3; &MCT5/L-arg5 vs. MCT5; $P < 0.05$. C: survival rate was significantly decreased in MCT-treated animals. L-Arg prolonged survival of MCT-treated animals. *MCT vs. Control; #L-Arg vs. MCT; $P < 0.05$. D: body weight decrease was evident at 3 and 5 wk post-MCT. L-Arg prevented body weight loss in MCT-treated animals. *MCT3 vs. Control 3 wk, MCT5 vs. Control 5 wk; #MCT3/L-arg3 vs. MCT3; &MCT5/L-arg5 vs. MCT5; $P < 0.05$. 

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3, A–H, display fluorescent microscopy images of the perfused lungs. Control lungs displayed low levels of O$_2^-$ as indicated by low-fluorescing nuclei in pulmonary cells. L-Arg itself had no effect on O$_2^-$ generation in lung. Treatment of rats with MCT dramatically increased 2-OH-Et$^+$ staining in the nuclei of pulmonary cells in the lungs both at 3 and 5 wk post-MCT. L-NAME and L-Arg both markedly decreased 2-OH-Et$^+$ staining in the nuclei of pulmonary cells at 3 and 5 wk post-MCT. Relative changes in O$_2^-$ as assessed by fluorescence intensity of Et in 10–20 animals, are shown in Fig. 3I.

**Effects of L-Arg on expression of eNOS, HSP90, and phosphorylation of eNOS at Ser$^{1177}$ in lung.** The influence of L-Arg on expression of eNOS and HSP90, and phosphorylation of eNOS at Ser$^{1177}$ in MCT-induced PH lung was assessed. The representative blot of β-actin in Fig. 4 shows that the amount of protein loading in each group was equal. The expression of eNOS and HSP90 and the phosphorylation of eNOS at Ser$^{1177}$ were markedly decreased in MCT-treated animals at both 3 and 5 wk compared with control lung. L-Arg could partially preserve eNOS expression and phosphorylation at Ser$^{1177}$ at both 3 and 5 wk post-MCT. Although L-Arg slightly increased HSP90 expression at 3 and 5 wk post-MCT, it did not reach statistical significance. The effects of L-Arg on the expression of eNOS and HSP90, and the phosphorylation of eNOS at Ser$^{1177}$ sites in lung can be seen in the bar charts showing the mean (n = 10) of relative band densities.

**Effects of L-Arg on association of HSP90 with eNOS in lung.** To determine the effects of L-Arg on eNOS protein interaction with HSP90 in MCT-induced PH lung, lungs were extracted for protein isolation and immunoprecipitated with eNOS. Immunoblots for eNOS and HSP90 on the immunoprecipitated eNOS complex revealed that the association of HSP90 with eNOS was significantly decreased with the development of PH compared with control lungs. (Fig. 5). L-Arg could partially restore the association of HSP90 with eNOS at 3 and 5 wk post-MCT. The effects of L-Arg on association of HSP90 with eNOS relative to controls are depicted in the bar chart (n = 10) showing the mean of relative band densities.

**Effects of L-Arg on pulmonary vascular remodeling.** As shown in Fig. 6, the wall thickness of fully muscularized distal pulmonary arteries increased with time post-MCT compared with controls. L-Arg partially inhibited MCT-induced vessel wall thickness of pulmonary arteries. L-Arg itself had no effect on vessel wall thickness of pulmonary arteries in the control rats.

**DISCUSSION**

MCT has been used experimentally by many researchers to cause PH in rats since this animal model can mimic clinical PH (16, 39). It has been shown that PH was significantly increased in 2 wk, and rats began to die in 4 wk after injection of MCT (16, 39). Thus, we can consider 3 wk post-MCT as the middle stage of PH and 5 wk post-MCT as a late stage of PH. Therefore, we chose 3 and 5 wk post-MCT as the study end points to explore the changes of molecules during the development of PH and the effect of L-Arg in our present study.

eNOS activity is crucial in the development of PH (6, 7, 10, 14, 18, 20, 46). We (18) previously showed that eNOS expression in the pulmonary artery and peripheral lung is significantly decreased and that the association of HSP90 with eNOS is dramatically reduced in pulmonary artery segments from newborn lambs with persistent PH. We further showed that the association of HSP90 with eNOS in ATP stimulation-cultured endothelial cells isolated from newborn lamb with persistent PH is inhibited in a manner that corresponds with decreased NO generation (18). A recent study reported that cultured endothelial cells isolated from pulmonary arteries of persistent PH of newborn lambs increase O$_2^-$ generation in response to ATP stimulation (17). However, no direct evidence is available for the relationship of eNOS activity, eNOS coupling activity, and the balance of NO and O$_2^-$ in the development of PH.

In the present study, we found that eNOS and HSP90 expression, eNOS phosphorylation, and the association of HSP90 with eNOS decreased with time in the MCT-induced PH lung in a manner corresponding to decreased NO generation and increased production of O$_2^-$ in the lung. This was accompanied by an elevated RVSP, impressive right heart hypertrophy, increased wall thickness of pulmonary arteries, loss of body weight, and increased mortality. We and others (9, 31, 35) have demonstrated that HSP90 is essential for eNOS-dependent NO production and that the inhibition of HSP90 with eNOS uncouples eNOS activity and increases eNOS-dependent O$_2^-$ production. Moreover, it was also demonstrated that eNOS phosphorylation at Ser$^{1177}$ is an immunological index of eNOS enzyme activation and electron flow (25). Thus, findings from our present study suggest that the development of PH may be through two pathways: decreasing eNOS and phosphorylation at the Ser$^{1177}$ site to reduce the eNOS activity and inhibiting the association of eNOS and HSP90 to uncouple eNOS activity. As a result, the balance of NO and O$_2^-$ becomes altered, and the pulmonary arteries are injured, with contraction and proliferation of pulmonary vascular smooth muscle. Finally, pulmonary vascular resistance increases and PH develops, leading to right heart hypertrophy and mortality increase. This scenario is entirely consistent with the present results.
Fig. 3. Effects of l-Arg on $\text{O}_2^-$ generation in lung as monitored by fluorescence microscopy ($\times$200 magnification). Pseudocolorized images show fluorescence intensity of ethidine staining in a representative lung section. 

A: baseline ethidine staining in control lung. 
B: ethidine staining was no different between control and l-Arg-treated animals. Ethidine staining was increased significantly at 3 wk (C) and 5 wk (F) post-MCT and was reduced both by nitro-l-arginine methyl ester (l-NAME; D and G) and l-Arg (E and H), confirming that $\text{O}_2^-$ generation is increased in pulmonary artery hypertension and was decreased by l-Arg. 

I: relative changes in $\text{O}_2^-$, as assessed by fluorescence intensity of ethidine in arbitrary units. *MCT3, MCT5 vs. Control; #MCT3/l-arg3 vs. MCT3; &MCT5/l-arg5 vs. MCT5; $P < 0.05$. 
Interestingly, although many changes of molecules were more and more severe with the development of PH, \( \text{O}_2^- / \text{H}_2\text{O}_2 \) generation seemed to increase more at 3 wk post-MCT than that at 5 wk post-MCT in the present study. This finding is similar to the report from Mathew’s group that \( \text{O}_2^- / \text{H}_2\text{O}_2 \) generation at 2 wk post-MCT was more than that at 4 wk post-MCT (16). It is possible that the lungs became more injured in the late stage of PH so that it could not generate as much \( \text{O}_2^- \) as in the middle stage of PH. Such findings suggest that the changes of different molecules may vary in different stages of PH.

L-Arg, which is the precursor of NO, can increase NO production and may be used as a therapeutic reagent for PH. Short-term and long-term infusion of L-Arg reduces PH, pulmonary vascular resistance, and endothelin release and increases plasma levels of L-citrulline in humans and animals (5, 27, 28). Oral administration of L-Arg can also lower PH and prevent right ventricular hypertrophy (38). A recent study reported that supplemental dietary L-Arg reduced PH mortality with elevated NO production in a cool-induced PH bird model (46). In this study, we tried to observe whether injection of L-Arg directly affected pulmonary NO and \( \text{O}_2^- \) generation in the development of PH. We found that L-Arg not only restored NO concentration but also reduced \( \text{O}_2^- / \text{H}_2\text{O}_2 \) generation in lung. In line with these findings, a decrease of RVSP, inhibition of right heart hypertrophy, reduction of pulmonary artery wall thickness, preservation of body weight, and increased survival rate were observed in the MCT-induced PH rat model. These data suggest that L-Arg may restore the NO and \( \text{O}_2^- / \text{H}_2\text{O}_2 \) balance to delay the development of PH. In addition, the fact that both L-NAME and L-Arg decreased \( \text{O}_2^- / \text{H}_2\text{O}_2 \) generation shows that an important part of the increased \( \text{O}_2^- \) generated in PH is due to eNOS uncoupling, since L-NAME inhibits the enzyme and L-Arg corrects the uncoupling.

It is well known that NO reacting with \( \text{O}_2^- / \text{H}_2\text{O}_2 \) can generate peroxynitrite (ONOO), a highly toxic molecule, which can produce more harmful effects. In the normal condition, the level of ONOO remains very low because of the low level of \( \text{O}_2^- \) generation. In PH, however, ONOO production increases
due to the $O_2^-\cdot$ generation enhancement (30, 48). Thus, the decrease of $O_2^-\cdot$ generation by L-Arg can reduce ONOO production and in turn limit the injury of lungs during the development of PH. Although the reaction between NO and $O_2^-\cdot$ is a significant source of ONOO production, other sources can also produce ONOO; therefore, future studies will be needed to determine whether L-Arg reduces ONOO production from other sources.

To further explore the molecular mechanism of L-Arg restoration of the NO and $O_2^-\cdot$ balance and attenuating PH, protein expression and association were examined. L-Arg partially prevented the reduction of eNOS expression and phosphorylation at Ser1177 without significantly altering HSP90 expression in lung post-MCT. Furthermore, L-Arg partially preserved the association of eNOS with HSP90 in the lung. Such findings indicate that L-Arg can maintain eNOS activity and eNOS coupling activity during the development of PH. Our data confirm the previous finding that L-Arg increases NOS expression in pulmonary endothelial cells with significantly increased plasma NO (46).

How the administration of L-Arg increases HSP90 associated with eNOS remains unknown. A previous study demonstrated that, besides serving as a substrate for NOS, L-Arg can also stimulate the release of insulin and histamine to induce vasodilation (11, 13, 36). Meanwhile, it has been shown that insulin can induce eNOS phosphorylation at Ser1179 and HSP90 associated with eNOS (45). As a calcium-mobilizing agonist, histamine has been shown to cause the binding of HSP90 to eNOS (9). Thus, it is possible that L-Arg may stimulate the release of insulin and histamine to promote the association of HSP90 with eNOS and eNOS phosphorylation to restore the NO and $O_2^-\cdot$ balance. Alternatively, L-Arg may induce extracellular acidosis, which, in turn, can transiently alter intracellular pH, thereby affecting pH-dependent cell-signaling pathways, including calcium transient pathways, that modulate eNOS activity and NO synthesis (22).

A recent study reported that lung L-Arg levels were decreased in a lamb PH model induced by increasing pulmonary blood flow (41). Those authors found that lung L-Arg levels correlated with an increase in NOS-derived reactive oxygen species generation. Such a study provides insight into other molecular mechanisms leading to decreased NO signaling and increased $O_2^-\cdot$ generation in PH and suggests that alterations in lung L-Arg metabolism may play a role in the development of the PH. These findings provide further direct evidence concerning how the administration of L-Arg can restore NO and $O_2^-\cdot$ balance and attenuate PH.

Another recent study reported that L-Arg can enhance angiogenesis in hypoxic pulmonary circulation (15). These findings suggest that one of the mechanisms by which L-Arg attenuates hypoxic pulmonary hypertension may be related to the development of new parallel vascular pathways through the lung (15). Both eNOS and HSP90 are critical for angiogenesis (2, 34, 44, 49); therefore, L-Arg may promote angiogenesis by preserving eNOS and the association of eNOS and HSP90 as well as eNOS phosphorylation.

It should be noted that some anesthetic agents including pentobarbital have been reported to cause hypotension, which may affect the measurement of RVSP (1). Although a lot of research has chosen pentobarbital-anesthetized animals in PH studies (2–6, 9, 10), to avoid this side effect induced by pentobarbital, all animals were anesthetized the same way in our study and the side effect should have been limited. Another way to avoid the pentobarbital effect is to use an agent other than pentobarbital in any future study.
In summary, our findings indicate that PH has a deleterious effect on pulmonary NO and O$_2^-$ balance and that decreased eNOS activity and uncoupled eNOS activity may play an important role in the alteration of this balance. L-Arg may act through three pathways to restore eNOS activity and eNOS coupling activity to maintain the balance between NO and O$_2^-$ and delay the development of PH. These pathways include providing a substrate of NO generation, preserving eNOS expression and phosphorylation, and maintaining the association of eNOS and HSP90. Our findings may provide important theoretical basis to further explore the pathogenesis, prevention, and therapeutic measures for PH. Future studies are required to determine the relative contribution of these pathways to the overall protection observed.

GRANTS

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DISCLOSURES

No conflicts of interest are reported by the authors.

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20. Leblais V, Delannoy E, Fresquet F, Begueret H, Bellance N, Waxman L. Enhanced electron flux through three pathways to restore eNOS activity and eNOS coupling activity to maintain the balance between NO and O$_2^-$ and delay the development of PH. These pathways include providing a substrate of NO generation, preserving eNOS expression and phosphorylation, and maintaining the association of eNOS and HSP90. Our findings may provide important theoretical basis to further explore the pathogenesis, prevention, and therapeutic measures for PH. Future studies are required to determine the relative contribution of these pathways to the overall protection observed.


