Raisanberine protected pulmonary arterial rings and cardiac myocytes of rats against hypoxia injury by suppressing NADPH oxidase and calcium influx

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Aim: To investigate the protection of pulmonary arterial rings and cardiac myocytes of rats by raisanberine (RS), a derivative of berberine, against hypoxia injury and to elucidate the action mechanisms.

Methods: Adult SD rats were exposed to intermittent hypoxia for 17 d or 28 d. The pulmonary arterial rings were isolated and vascular activity was measured using a transducer and computer-aided system. The difference in the tension produced by phenylephrine in the presence and absence of L-nitroarginine (10 μmol/L) was referred to as the NO bioavailability; the maximum release of NO was assessed by the ratio of the maximal dilatation caused by ACh to those caused by sodium nitroprusside. After the lungs were fixed, the internal and the external diameters of the pulmonary arterioles were measured using a graphic analysis system. Cultured cardiac myocytes from neonatal rats were exposed to H2O2 (10 μmol/L) to mimic hypoxia injury. ROS generation and [Ca2+]i level in the myocytes were measured using DHE and Fluo-3 fluorescence, respectively.

Results: Oral administration of RS (80 mg/kg), the NADPH oxidase inhibitor apocynin (APO, 80 mg/kg) or Ca2+ channel blocker nifedipine (Nif, 10 mg/kg) significantly alleviated the abnormal increase in the vasoconstriction force and endothelium-related vasodilatation induced by the intermittent hypoxia. The intermittent hypoxia markedly decreased the NO bioavailability and maximal NO release from pulmonary arterial rings, which were reversed by APO or RS administration. However, RS administration did not affect the NO bioavailability and maximal NO release from pulmonary arterial rings of normal rats. RS, Nif or APO administration significantly attenuated the pulmonary arteriole remodeling. Treatment of cultured cardiac myocytes with RS (10 μmol/L) suppressed the ROS generation and [Ca2+]i increase induced by H2O2, which were comparable to those caused by APO (10 μmol/L) or Nif (0.1 μmol/L).

Conclusion: Raisanberine relieved hypoxic/oxidant insults to the pulmonary artery and cardiac myocytes of rats by suppressing activated NADPH oxidase and increased calcium influx.

Keywords: raisanberine; apocynin; nifedipine; hypoxia; pulmonary artery; cardiac myocytes; NADPH oxidase; calcium influx

Introduction

Pulmonary arterial hypertension (PAH) caused by hypoxia is characterized by a progressive elevation of the resistance of the pulmonary arterial system and insults to the heart; hypoxia causes oxidant lesions that adversely affect cardiac myocytes, contributing in part to right heart failure and increased mortality[1]. The pulmonary vascular endothelium is abnormal after exposure to hypoxia, elevating the pulmonary arterial pressure by enhancing constriction and reducing dilatative activity due to compromised NO availability. Extensive research demonstrates that an excess of ET-1 (endothelin-1) is actively involved in the pathogenesis of PAH and that PAH can be reversed by ET receptor-blocking agents[2, 3].

Endothelium-dependent relaxation depends on the biosynthesis and release of NO by endothelial nitric oxide synthase (eNOS) in the vascular endothelia. The activity of eNOS generates both NO and superoxide (O2-), which are key mediators of cellular signaling in regulating vascular activity[4]. Vascular relaxation by NO is counteracted by activated ETα, resulting in compromised NO bioavailability and a decrease in the eNOS activity, which are related to an excess of reactive oxygen species (ROS), and is likely attributable to activated NADPH oxidase. ET-1 is an inducer of NADPH oxidase mainly through ETα[5], and the biosynthesis and release of the vascular dilating substance, NO, in vascular endothelia are seriously affected by oxidative stress[6]. The pathogenesis of hypoxic PAH can be characterized by an activated ET pathway, which was relieved
by the ET antagonists CPU0213[7] and CPU86017[8], and its iso-
mers[9] by reducing ETα expression in the pulmonary vascula-
ture.

NADPH oxidase-derived ROS may play a major role in the
pathology of hypoxia-induced PAH[10]. NADPH oxidase
belongs to a family of proteins that consist of two groups of
subunits, catalytic components, NOX1, gp91phox (NOX2), and
NOX4, and modulating components, p22phox, p47phox, and
p67phox, which localize separately either at the membrane or
in the cytosol[11]. Decreased vasorelaxation is mediated by an
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Raisanberine (RS compound, an isomer of CPU86017: [+]-7R, 13αS-p-chloro-benzyl-tetra-hydro-berberine chloride)
is active in reversing the abnormal expression of the ET sys-
tem in pulmonary artery hypertension in rats[15, 16]. In our
previous study, RS improved the remodeling of the pulmo-

We hypothesized that an activated NADPH oxidase may be
caused by hypoxia and induce abnormal pulmonary arter-

ti activity and intracellular free calcium in cardiac myocytes.
H2O2-induced abnormalities of intracellular calcium in cardiac
myocytes mimic the insults due to hypoxia and are likely due
to an activation of NADPH oxidase. The aim of our research
was to verify this hypothesis by employing apocynin, an
inhibitor of NADPH oxidase, the calcium channel blocker nife-
dipine, and RS to test their efficacy in alleviating the hypoxia-
related abnormalities of pulmonary arteries and cardiac myo-
cytes.

Materials and methods

Experimental animals

Male Sprague-Dawley rats, weighing 220–250 g, were pur-
chased from the Experimental Animal Center of Zhejiang
Province, Hangzhou. License Ng: SCXK (Zhe) 20080033. The
procedures involving experimental animals were conducted
by personnel trained according to the Experimental Animal
Handling Act instituted by the Science-Technology Bureau of
Jiangsu Province, China.

Drugs and reagents

Raisanberine (CPU86017-RS, RS) was synthesized and pro-
vided by the Department of Medicinal Chemistry, China Phar-
maceutical University, Lot 20090301. Nifedipine (Nif) (10 mg/
tablets) was obtained from Changzhou Kangpu Pharmaceutical
Co, Ltd, Lot 0910004. Apocynin (APO) (white crystalline
powder; Lot 122F20340) was obtained from Sigma-Aldrich
Chemie GmbH, Steinheim, Germany. DHE and Fluo-3/AM
were purchased from Sigma-Aldrich, St Louis, MO, USA.

Exposure to intermittent hypoxia

A total of 110 rats were randomly divided into two experi-
ments: (1) in Exp 1, 4 groups (n=6) were exposed to intermit-
tent hypoxia for 17 d, and in the last 12 d, APO 80 mg/kg and
RS 80 mg/kg was administered (po); in Exp 2, hypoxic rats
were untreated (Hyp) or treated (mg/kg, po) with Nif (10),
APO (80), RS low dose (RSL, 20), RS medium dose (RSM,
40), or RS high dose (RSH, 80), and normal rats were either
untreated (Nor) or received the RS high dose (N+RS, 80); these
8 groups (n=10) received the treatments in the last 14 d of
the experiment. According to a previous report[8] we used a
hypoxia chamber in which the oxygen fraction was main-
tained at 10%±0.5% by controlling the N2 flow into the chamber.
The water vapor and carbon dioxide were removed by placing
anhydrous calcium chloride and sodium lime in the chamber.
The rats were kept in the hypoxic chamber for 8 h per day for
17 and 28 d for Exp 1 and 2, respectively, except for the Nor
and N+RS groups. The rats in the Nor, Hyp and N+RS groups
were administered the same volume of 0.5% CMC-Na.

Vascular activity of isolated pulmonary arterial rings

The rats were anesthetized with urethane 1.5 g, ip, the chest
was opened, and the pulmonary artery was rapidly harvested
and placed in cold K-H solution, containing the following
(in mmol/L): NaCl 119, NaHCO3 25, KCl 4.6, KH2PO4 1.2,
MgCl2 1.2, CaCl2 2.5, and glucose 11, and saturated with 95%
O2+5% CO2 (2 bubbles/s). The connective tissue surrounding
the artery was removed, and the pulmonary artery was cut
into rings of 2 mm in width. The rings were fixed by a trian-
gular steel ring to the bottom of a 3 mL organ bath and con-
ected to a transducer and a computer-aided system for real
time recordings, as previous reported[8]. The resting tension
baseline of the vascular ring was loaded to 0.6–0.7 g. After sta-
bilization for 2 h with 3 tests of norepinephrine at 1 µmol/L,
Phe (phenylephrine, 3, 30, and 300 nmol/L) was added in
sequence for recordings of the developed tension of the vas-
cular rings. After reaching the plateau, the isolated vascular
rings were dilated by adding ACh (acetylcholine, 0.01, 0.1, 1,
and 10 µmol/L), and the vascular rings were dilated further by
adding SNP (sodium nitroprusside, 10 µmol/L) until reaching
the maximum relaxation. After incubating the vascular rings
for 15 min with L-NA (L-nitroarginine, an inhibitor of eNOS,
10 µmol/L), the testing of vascular contraction and relax-
ation was repeated. The difference in the tension produced
by Phe in the presence and absence of L-NA was taken as the
functional assessment of the NO released from the vascular

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endothelium and referred to as the NO bioavailability; the maximum release of NO was assessed by using a functional approach. The relaxation caused by ACh is endothelium dependent and can be considered to represent the maximum release of NO. The ACH-induced relaxation was compared to those caused by SNP which was taken as 100%, therefore, the ability of the pulmonary artery to release NO could be calculated\[13, 18\].

**Morphological analysis of pulmonary arterioles**

The lungs were fixed with neutral formalin, embedded in paraffin, and sliced into 4-μm-thick sections (HE stain, ×400). The length of the internal (ID) and the external diameter (ED) of the pulmonary arterioles were measured using a graphic analysis system (IMAGE-PROPLUS [Media Cybernetics, Silver Spring, MD])\[9\]. The pulmonary arterioles with EDs less than 150 μm were selected, and 10 visual fields for each slide were randomly chosen.

The wall thickness (WT) percentage was calculated as follows:

\[
WT(\%) = 100 \times 0.5 \times (ED-ID)/ED[^9].
\]

**ROS in cultured cardiac myocytes**

The cardiac myocytes from neonatal rats (48 h) were employed for assessing the fluorescence intensity of ROS when stained with DHE (dihydroethidium), as based on a previous report\[19\]; for assessing the fluorescence intensity of ROS when stained, the cell density was adjusted to 6×10^5/mL and diluted 3-fold. After adding CaCl_2 (10 mmol/L) and A23187 (5 mmol/L) into Tyrode’s solution, the fluorescence intensity was abruptly decreased to a certain level and remained unchanged; this was recorded and referred to as F_{max}. The levels of [Ca^{2+}], were calculated according to the following formula: [Ca^{2+}] = (F_{max} - F)/Kd. The peak (systolic) and trough (diastolic) concentrations of [Ca^{2+}], of the beating cardiac myocytes were obtained, and the difference between the two was considered a “change” in the intracellular calcium concentrations in the cardiac cycles. These data were compared among the groups.

**Statistical analysis**

All of the data were expressed as the mean±SD. Student’s t-test was used to determine the significant differences between two groups, and a one-way ANOVA was used to evaluate three or more groups. Excel and the Graph Pad Prism 5 programs were used for the statistical analysis, and a value of P<0.05 was considered significant.

**Results**

**An increase in constrictive force**

Phe was added successively (3, 30, and 300 nmol/L) to induce constriction in the pulmonary arterial rings of the normal and hypoxic groups, and the increase in the developed force was calculated (P<0.01) in the hypoxic group in Exp 1 and 2 compared with the normal group (Figure 1A–1D). Hypoxia exposure clearly enhanced the constrictive force of the pulmonary arterial rings, thereby increasing vascular resistance in the pulmonary arterial system. The increased vascular resistance caused hypoxia pulmonary artery hypertension. The abnormal increase in the vasocontractile force was reduced (P<0.01) by APO, Nif, and RS (Figure 1). RS did not affect the constrictive activity of the normal pulmonary arterial rings.

**Impaired endothelium-dependent relaxation**

After the vasconstriction caused by Phe reached a plateau, ACh was added successively (0.01, 0.1, 1, and 10 μmol/L) to relax the pulmonary arterial rings. The endothelium-related vasodilatation was significantly compromised (P<0.01) in the hypoxic rats compared to the normal rats and was significantly alleviated by APO, Nif, and RS in Exp 1 and 2 (Figure 2). In contrast, following the addition of SNP 10 μmol/L, the vasorelaxation of vascular smooth muscle was dilated directly and was not impaired in the hypoxic group; therefore, no difference was found among the groups. RS did not cause an increase in the ACh-induced vasorelaxation in normal rats.

**Reduced NO bioavailability and maximal NO release**

The NO bioavailability was evaluated by vascular activity assessment of the pulmonary arterial rings using two procedures. First, the constrictive responses to three concentrations of Phe were monitored, and the AUC (area under the curve) of the vasoconstrictive force was calculated. Second, the AUC was estimated again after L-NA, an inhibitor for eNOS, was added in a separate experiment. The difference of the AUC in the presence and absence of L-NA represents the NO bio-
availability: the larger the difference between the two AUC values, the higher the NO availability in the tested artery. The increased constrictive force of the hypoxic pulmonary artery rings in the absence of L-NA was due to reduced NO availability in the pulmonary artery, and a smaller increase in the contractile force was observed while L-NA was added. NO bioavailability was decreased significantly in the Hyp group (P<0.01 vs Nor) in these two experiments and was remarkably increased (P<0.01 vs Hyp) after APO and RS interventions. RS did affect the NO bioavailability of the normal group (P>0.05) (Figure 3A, 3C).

Both in Exp 1 and 2, the maximal NO release was decreased significantly in the Hyp group (P<0.01 vs Nor) in these two experiments and was remarkably increased (P<0.01 vs Hyp) after APO and RS interventions. RS had no effects on the maximum NO release of the normal group (P>0.05) (Figure 3B, 3D).

Remodeling of the pulmonary arterioles
Changes in vascular activity are always associated with a morphological remodeling of the pulmonary vasculature; therefore, the changes in the thickness of vascular smooth muscle in the pulmonary arteriole were examined. A wall thickness (WT) of the pulmonary arterioles less than 150 µm in diameter was evaluated in Exp 1 and 2, and a remodeling of the vasculature, characterized as increased vascular wall thickness, was significant (P<0.01) in the hypoxic rats when compared with the normal rats in Exp 2 (Figure 4A). Similar data from Exp 1 are not shown. Treatment with Nif, APO, and RS (20, 40, and 80 mg/kg) attenuated the pulmonary arteriole remodeling compared to the Hyp group. The reduction of the arteriolar vasculature thickness by RS occurred in a dose-dependent manner, and the RS 80 mg/kg alone did not affect the WT of the pulmonary arterioles in the normal rats (Figure 4A, 4C).

Suppression of increased ROS in cultured cardiac cells
ROS generation was enhanced in the cardiac myocytes incubated with H₂O₂, which was suspected to activate NADPH oxidase in the myocytes. Accordingly, we added APO to the medium to determine whether APO could suppress the ROS generation, as labeled with DHE fluorescence in the cardiac cells; we also addressed whether direct evidence of ROS-suppressing activity for RS could be found. Hypoxia damages cells mainly by increasing ROS generation; therefore, isolated cardiac cells of neonate rats were cultured with H₂O₂ (10 µmol/L), which mimics the injury from hypoxia exposure.
The fluorescence was significantly exaggerated in the H$_2$O$_2$ group ($P<0.01$), as compared with the normal group, and the exaggerated ROS generation was significantly suppressed ($P<0.01$) by APO, an inhibitor of NADPH oxidase. Interestingly, the beneficial effects of Nif were also significant. The suppressive effects of RS on ROS generation were positive and comparable to those of APO and Nif (Figure 4B, 6B).

**Suppression of intracellular [Ca$^{2+}$]$_i$**

To evaluate the suppression of [Ca$^{2+}$]$_i$, by APO and RS, the isolated primary cardiac myocytes were cultured with H$_2$O$_2$ to mimic hypoxia injury. An abnormal increase of [Ca$^{2+}$]$_i$ was found in the beating cardiac myocytes with Hyp (hypoxia that was mimicked by adding H$_2$O$_2$, $P<0.01$), as compared with the normal group). The systolic and diastolic values and their changes were significantly enhanced after exposure to H$_2$O$_2$, highlighting the likely involvement of an increase in the intracellular calcium in hypoxic pulmonary arteries. The abnormal increase of intracellular calcium was greatly reduced ($P<0.01$) by the intervention with Nif; however, the effects were reproduced by an NADPH oxidase blocker, APO. RS was found to reduce the enhanced [Ca$^{2+}$]$_i$, caused by hypoxia, which was comparable to that of Nif and APO (Figure 4C, 5, 6C).

**Discussion**

We reported previously that the pulmonary artery is more sensitive to hypoxia than the systemic circulation[22]. Chronic hypoxia exaggerates the production of ROS, which likely occurs in multiple steps. ROS are primarily produced from the abnormal electron transport in the mitochondria, and the produced ROS in turn stimulate NADPH oxidase to cause further and sustained ROS production (Figure 7). An excessive production of ET-1, a causal factor contributing to pulmonary hypertension, is stimulated by ROS; in fact, ETA and ETB receptors are actively implicated in the pulmonary hypertension caused by either hypoxia[7, 9] or the inflammatory substance, monocrotaline[8, 23]. Emerging data suggest that endothelin receptor antagonists, either selective ETA blockers or dual ETA and ETB blockers, successfully alleviate pulmonary hypertension in both experimental animals and clinical settings[23, 24]. Therefore, ET-1 has been considered the most important factor affecting the pulmonary arterial system and critically impairs vascular activity. In addition, potent vasoconstrictive activity, eg, an activated ET$_A$, reduces the NO bioavailability and maxi-
mal NO release from the vascular endothelium, thus exacerbating vascular resistance and remodeling in pulmonary circulation.

The bioactivity of ET-1 is at least in part mediated by NADPH oxidase, these including positive inotropism in the heart and an elevated \([\text{Ca}^{2+}]_i\), which is due to an increased frequency of open individual L-type channels; therefore, increased calcium influx is a consequence of the activation of \(\text{ET}_{\alpha}\) and the activation of L-type channels by \(\text{ET}_A\) is mediated by the activation of NADPH oxidase\(^{[25, 26]}\) (Figure 7).

Activated NADPH oxidase is the main source of ROS production, which is evidenced by the appearance of vascular abnormalities upon exposure to intermittent hypoxia and \(\text{H}_2\text{O}_2\)-induced ROS and an increase in the intracellular calcium in cardiomyocytes. NADPH oxidase as an important source of excessive ROS production, providing insight into many diseases, including diabetic nephropathy and cardiomyopathy and pulmonary hypertension\(^{[27-29]}\). In general, an activated NADPH oxidase produces ROS under hypoxic conditions; conversely, ROS serve as important inducers of ET-1, angiotensin, and tumor necrosis factor \(\alpha\), which stimulate NADPH oxidase. Our data are in agreement with our previous report that NADPH oxidase is an important source of ROS production in cardiac fibroblasts\(^{[5]}\).

The calcium balance in the pulmonary vascular cells critically modulates the vasoactivity of the pulmonary artery, and an increase in \([\text{Ca}^{2+}]\), allows an increase in the vascular tone responding to vasoconstrictive agents. An intracellular release of calcium from RyRs (ryanodine receptors) in vascular smooth muscles\(^{[30]}\) occurs in response to calcium influx through L-type channels, which is exacerbated by an enhanced influx of calcium, resulting in an increase in \([\text{Ca}^{2+}]_i\), in the systolic and diastolic cycles. However, it has been reported that the removal of extracellular \(\text{Ca}^{2+}\) may not affect the \(\text{H}_2\text{O}_2\)-induced increase in \([\text{Ca}^{2+}]_i\)\(^{[18]}\), indicating that a direct effect of stimulating intracellular \(\text{Ca}^{2+}\) release could be involved. In the present study, the application of nifedipine, which inhibits calcium influx, was expected to suppress the increased calcium levels by limiting the calcium ions transporting into the cells. L-type calcium channel activation by \(\text{H}_2\text{O}_2\) (ROS) often follows hypoxemia after the exposure to intermittent hypoxia. In the present study, the increased calcium influx was suppressed by nifedipine and attenuated by the inhibition of NADPH oxidase. We provide direct evidence demonstrating that an activation of NADPH oxidase is also involved in exacerbated calcium influx; thus, by suppressing NADPH oxidase, apocynin treatment leads to a reduction in \([\text{Ca}^{2+}]_i\) in the cardiac myocytes. Therefore, as measured by an increase in ROS generation, we provide evidence that the activity of NADPH oxidase is linked to significant calcium influx, implying that a decline in \([\text{Ca}^{2+}]_i\), reduces ROS generation, thus preventing the activation of NADPH oxidase under hypoxia\(^{[30]}\). The NADPH oxidase blocker apocynin sufficiently normalizes the exaggerated intracellular calcium in cardiomyocytes. We emphasize that NADPH oxidase modulates the free calcium levels in cardiac myocytes and, thus, participates in the pathogenesis of the cardiomyopathy caused by hypoxemia and contributes to hypoxic pulmonary hypertension (Figure 7).

In conclusion, abnormal vascular activity and the remodeling of pulmonary arterioles are due to the activation of NADPH oxidase under hypoxia, which is linked to activated...
etA and a reduction in the bioavailability of NO. Exacerbation of the intracellular calcium levels by H2O2 to mimics injury due to hypoxia and can be blocked by apocynin, an inhibitor of NADPH oxidase. Therefore, the inhibition of NADPH oxidase attenuates both the abnormal pulmonary vascular activity and the increase in ROS generation in cardiac myocytes. These abnormalities are normalized by raisanberine, which presumably suppresses the calcium influx and inhibits NADPH oxidase in the pulmonary arteriole and cardiac cells, respectively.

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Author contribution
Jie GAO conducted the project and prepared the manuscript. Yu-si CHENG assisted in performing the cellular experiments. Guo-lin ZHANG and Yi-qun TANG collected and processed data. Yin DAI supervised the experiments. Can ZHANG prepared and provided the tested compound. The project design, analyses of the results and revision of the manuscript were performed by De-zai DAI.

References
1 Hernandez-Rivera H, Martinez-Gonzalez H, Casal J, Rodriguez-Ospina L. Pulmonary arterial hypertension: from pathophysiology to management. Bol Asoc Med P R 2011; 103: 34–40.
2 Yuan SH, Dai DZ, Guan L, Dai Y, Ji M. CPU0507, an endothelin receptor antagonist, improves rat hypoxic pulmonary artery hypertension and constriction in vivo and in vitro. Clin Exp Pharmacol Physiol
3 Dupuis J, Hoeper MM. Endothelin receptor antagonists in pulmonary arterial hypertension. Eur Respir J 2008; 31: 407–15.
4 Chen CA, Wang TY, Varadharaj S, Reyes LA, Hemann C, Talukder MA, et al. S-glutathionylation uncouples eNOS and regulates its cellular and vascular function. Nature 2010; 468: 1115–8.
5 Peng HJ, Dai DZ, Ji H, Dai Y. The separate roles of endothelin receptors participate in remodeling of matrix metalloproteinase and connexin 43 of cardiac fibroblasts in maladaptive response to isoproterenol. Eur J Pharmacol 2010; 634: 101–6.
6 Dai DZ, Dai Y. Role of endothelin receptor A and NADPH oxidase in vascular abnormalities. Vasc Health Risk Manag 2010; 6: 787–94.
Figure 7. The mechanisms underlying the abnormal vascular activity in hypoxic pulmonary artery. Hypoxia promotes the genesis of ROS from the electron transport chain (ETC) at mitochondria, and thereafter, increases ET-1. ET-1 activates NADPH oxidase which contains catalytic (NOX1-4) and modulating subunits (p22phox, p47phox, and p67phox). An increased ROS stimulates the L-type Ca\(^{2+}\) channels to increase calcium influx and subsequent intracellular Ca\(^{2+}\) release from RyRs. Increased [Ca\(^{2+}\)] in turn stimulates the formation of ROS. ROS serves as an inducer for activating NADPH oxidase, therefore, blocking calcium influx is able to suppress NADPH oxidase. An increase in ET-1 and NADPH oxidase play key roles in the development of hypoxia pulmonary hypertension. A reduction in NO availability and release contributes to abnormal activity of hypoxic pulmonary artery. CPU86017-RS relieves hypoxic pulmonary arterial abnormality via blocking NADPH oxidase and calcium influx in the vasculature.
23 Cui B, Cheng YS, Dai DZ, Li N, Zhang TT, Dai Y. CPU0213, a non-selective ETA/ETB receptor antagonist, improves pulmonary arteriolar remodeling of monocrotaline-induced pulmonary hypertension in rats. Clin Exp Pharmacol Physiol 2009; 36: 169–75.

24 Galiè N, Negro L, Simonneau G. The use of combination therapy in pulmonary arterial hypertension: new developments. Eur Respir Rev 2009; 18: 148–53.

25 De Giusti VC, Correa MV, Villa-Abrille MC, Beltrano C, Yeves AM, de Cingolani GE, et al. The positive inotropic effect of endothelin-1 is mediated by mitochondrial reactive oxygen species. Life Sci 2008; 83: 264–71.

26 Zeng Q, Zhou Q, Yao F, O’Rourke ST, Sun C. Endothelin-1 regulates cardiac L-type calcium channels via NAD(P)H oxidase-derived superoxide. J Pharmacol Exp Ther 2008; 326: 732–8.

27 Xu J, Yu F, Dai DZ, Dai Y. High glucose-induced vascular dysfunction and expression of vascular ET_AR and OB-Rb: intervention effects of ETR antagonist CPU0213. Prog Pharm Sci 2009; 3: 125–31.

28 Dennis KE, Aschner JL, Milatovic D, Schmidt JW, Aschner M, Kaplowitz MR, et al. NADPH oxidases and reactive oxygen species at different stages of chronic hypoxia-induced pulmonary hypertension in newborn piglets. Am J Physiol Lung Cell Mol Physiol 2009; 297: L596–607.

29 Sedeek M, Caliera G, Montezano A, Gutsol A, Heitz F, Szondralewiez C, et al. Critical role of Nox4-based NADPH oxidase in glucose-induced oxidative stress in the kidney: implications in type 2 diabetic nephropathy. Am J Physiol Renal Physiol 2010; 299: F1348–58.

30 Wang YX, Zheng YM. ROS-dependent signaling mechanisms for hypoxic Ca\(^{2+}\) responses in pulmonary artery myocytes. Antioxid Redox Signal 2010; 12: 611–23.

31 Amberg GC, Earley S, Glapa SA. Local regulation of arterial L-type calcium channels by reactive oxygen species. Circ Res 2010; 107: 1002–10.