— Regular Article —

Dichloroacetate Inhibits Vascular Remodeling and Increases Voltage-gate K^+ Expression in a High-altitude-induced Pulmonary Artery Hypertension Rat Model

Xian-hua Xu, Hua-sheng Peng, Mei-qin Sun, Meng Hu, Wan-hua Wang, Ru Zhang, and Xin-rong Xiao*

Department of Geriatrics, Chengdu Military General Hospital, No. 270 Rongdu Road, Tian Hui Town, Chengdu, Sichuan Province 610083, P. R. China

(Received June 8, 2010; Accepted September 13, 2010)

To investigate the effect of dichloroacetate (DCA) on the mean pulmonary artery pressure (mPAP), pulmonary artery (PA) remodeling and voltage-gate K^+ (Kv) channel expression in pulmonary arterial smooth muscle cells (PASMCs) in high altitude-induced pulmonary artery hypertension (HA-PAH) rats. Sprague-Dawley rats were randomly assigned to normal control (N), high altitude (HA), and HA+DCA (70 mg/kg DCA administration daily) groups (n = 8 each). Rats were housed in a hypobaric, hypoxic chamber to mimic an altitude of 5000 m for 21 days; then the mPAP and the wall thickness (WT) of the PA smooth muscle were measured. PASMCs apoptosis was examined using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) stain. Real-time PCR, immunohistochemistry and western blot analyses were carried out to detect Kv1.5 and Kv2.1 expression in PASMCs. The expression of Kv1.5 and Kv2.1 was decreased in HA rats. With DCA treatment, the expression of Kv1.5 and Kv2.1 was restored, and the established HA-PAH was ameliorated. Compared with the HA-PAH rats, the DCA-treated rats displayed a decreased mPAP, WT of the PAs, right ventricular hypertrophy and ($[Ca^{2+}]i$), and more PASMCs were apoptotic. DCA partially reversed the down-regulation of Kv1.5 and Kv2.1 in the PASMCs of HA-PAH rats. DCA can reverse the remodeling of the PA and upregulate Kv1.5 and Kv2.1 expression in the PASMCs of HA-PAH rats. This result suggests that DCA may be an effective drug for treating HA-PAH and that restoring Kv1.5 and Kv2.1 can partially decrease mPAP.

Key words —— high altitude, pulmonary artery hypertension, dichloroacetate, vascular remodeling, voltage-gate K^+

INTRODUCTION

High altitude (HA) conditions involve hypobaric and hypoxic components. The air pressure and partial pressure of oxygen (PO_2) decrease as the altitude increases. Exposure of animals or humans to altitudes over 3000 m leads to the development of chronic high altitude-induced pulmonary artery hypertension (HA-PAH). This condition is an important pathological result of mountain sickness, and prolonged PAH results in right heart failure.[1] The mechanism of how PAH develops remains unknown, and the treatments for PAH are limited.

PAH is characterized by pulmonary arterial vasoconstriction (PAV) and pulmonary arterial remodeling (PAR), which result from an imbalance between the proliferation and apoptosis of smooth muscle cells (SMCs).[2] Although the endothelium is important in the pathogenesis of PAH, the role of vascular SMCs is paramount. Both the contractile and proliferative status of SMCs are regulated by the level of intracellular Ca^{2+}($[Ca^{2+}]i$).[3,4] Hypoxia causes a rapid and reversible increase in $[Ca^{2+}]i$, which leads to SMC contraction and proliferation. $[Ca^{2+}]i$ is determined in part by the influx of Ca^{2+} through voltage-gated, L-type Ca^{2+} channels,[5,6] which are gated based on the membrane

*To whom correspondence should be addressed: Department of Geriatrics, Chengdu Military General Hospital, No. 270 Rongdu Road, Tian Hui Town, Chengdu, Sichuan Province 610083, P. R. China. Tel. & Fax: +86-28-86570405; E-mail: xiaoxinrong1963@yahoo.com.cn

potential (Em) of the SMCs.[3,7]

In pulmonary arterial smooth muscle cells (PASMCs), the Em is partially regulated by voltage-gate K^+ (Kv) channels, and blocking Kv channels causes increased $[Ca^{2+}]i$.[3,7–9] Each Kv is composed of both pore-forming α- and β-subunits. The Kv α-subunits, including Kv1.2, Kv1.5, Kv2.1, and Kv9.3, are O_2-sensitive channels and are expressed in PASMCs.[10–13] Exposure to acute or chronic hypoxia, either *in vitro* or *in vivo*, results in decreased Kv channel activity, which is directly correlated with the downregulation of Kv1.2, Kv1.5, and Kv2.1.[13,14] Moreover, Kv2.1 regulates the basal Em,[3,8] whereas gene transfer of Kv1.5 attenuates hypoxia-induced PAH.[14] In monocrotaline-induced PAH rats, both Kv1.5 and Kv2.1 are also downregulated.[15] Thus, these two channel subtypes exhibit O_2-dependent expression and play an important role in the development of PAH.

Dichloroacetate (DCA), an orally available metabolic modulator, has been used extensively in humans to treat mitochondrial diseases and lactic acidosis and can enhance oxidative phosphorylation by inhibiting mitochondrial pyruvate dehydrogenase kinase.[16,17] DCA prevents and reverses chronic normobaric hypoxia and monocrotaline-induced PAH in rats.[15] However, little is known about its role in HA-PAH.

MATERIALS AND METHODS

Animals and Experimental Design —— All of the animal experiments were performed according to the Guidelines for Animal Care published by the Chinese government and the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institute of Health. Adult male Sprague-Dawley rats (200–250 g) were randomized into three groups ($n = 8$ each): normal control group (N), HA group and HA+DCA group. An altitude of 5000 m was simulated by exposing rats to a hypobaric, hypoxic chamber for 21 days as previously described.[18] Rats in the N group were housed under normal air pressure and PO_2 conditions. In the HA+DCA group, rats were given DCA (70 mg/kg per day, 20 mg/ml, pH 7.0, Sigma-Aldrich, Louis, MO, U.S.A.) on the first day of the simulated HA until the end of the experiments. The same volume of normal sodium was used in both the HA group and the N group. Food intake and body weight were measured for each rat before HA exposure and at days 3, 7, 14, and 21 after HA exposure.

Hemodynamic Measurements —— After 21 days in the simulated 5000-m altitude chamber, the rats were anesthetized with 10% urethane. The pulmonary artery (PA) pressure was measured with a 1.4-F catheter inserted into the right jugular vein and connected to a pressure transducer. Recordings were obtained using an analog-to-digital converter. The systemic blood pressure was recorded with a 1.4-F catheter inserted into the left common carotid artery, as previously described.[16] The HA and HA+DCA rats were measured in the simulated 5000-m, altitude chamber, whereas the N rats were measured under the normal conditions.

Histology Measurements —— The weight ratio of the right ventricle (RV) to the left ventricle (LV) plus septum (S) (RV/LV+S) was measured to determine right ventricular hypertrophy (RVH). The wall thickness (WT) was defined as the wall thickness of the small PAs (< 150 μm), and WT% is expressed as the percentage of wall thickness/external diameter × 100% of the small PAs. The wall area (WA) was defined as the wall area of the small PAs, and WA% is expressed as the percentage of wall area/whole small PAs area. The WT, WT%, WA, and WA% were measured to quantify the remodeling of the small PAs. Morphological analysis of the pulmonary arterioles was performed using IMAGE-PRO plus 6.0.

Free Ca^{2+} in SMCs in the Small Pulmonary Arteries —— Frozen sections were generated immediately after removal of the PA from the rats,[19,20] and Fluo-3-acetoxymethyl ester (AM) (Roche Diagnostics GmbH, Mannheim, Germany) was used to stain the free Ca^{2+} present in the SMCs of the small PA following the manufacturer's instructions. In brief, 50 μg of Fluo-3-AM was diluted in 45 μl dimethyl sulfoxide (DMSO) and then further diluted in 50 mM HEPES buffer to prepare the 20 μM working solution. After a 30-min incubation at room temperature, the slides were washed and observed under a fluorescence microscope (Olympus CK4032NFL, Olympus, Tokyo, Japan).

Quantitative PCR Evaluation of Kv mRNA Expression in PAs —— Rat pulmonary arteries were isolated for RNA extraction. Total RNA from the PAs was isolated from pooled segments of 21-day rats with an RNeasy mini kit (Takara, Dalian, China) following the manufacturer's protocol. The RNA content and purity were assayed by spectroscopy. 5 μg of total RNA sample was reverse-

transcribed into cDNA with random hexamers and Multiscribe Reverse Transcriptase (Takara). All of the primers and probes employed in the study for real-time PCR were designed using Primer Express Software (Bio-Rad iCycler, Hercules, CA, U.S.A.). The sequences of the Kv1.5 (Kcna5) primers were as follows: forward, 5′-CCT CCG ACG TCT GGA CTC AAT AA-3′; reverse, 5′-CCT CAT CCT CAG CAG ATA GCC TTC-3′; Kv2.1 (Kcnb1) primers: forward, 5′-ACA CGG GAG CAC TAG GGA TCA G-3′; reverse, 5′-CTC AGT GGC AGC AAG CCA AG-3′. Real-time PCR was conducted with a Premix EX Taq Kit (Takara) using the iCycler iQ Sequence Detection System (Bio-Rad iCycler). The N group was used as the endogenous control. The PCR cycling conditions consisted of an initial reverse transcriptase step at 50°C for 15 min, followed by a 2-min hold at 94°C, and then 45 cycles of 30 s at 55°C and 1 min at 72°C, and a final 5 min at 72°C.

Western Blotting to Measure Kv Protein Expression in the PA —— Isolated PAs were homogenized at 20000 rpm in 0.5 ml of HEPES buffer containing 10 nM HEPES, 1 nM dithiothreitol (DTT), and 1 tablet of protease inhibitor complex (Pierce, Rockford, IL, U.S.A.). The protein concentration was determined by the bicinchoninic acid protein assay. For each sample, 20 μg of total protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels and transferred to a Hybond-C extra nitrocellulose membrane (Bio-Rad, Hercules, CA, U.S.A.). The membranes were blocked with 5% nonfat dry milk and probed with rabbit polyclonal antibodies specific for various Kv channels (Santa Cruz, Santa Cruz, CA, U.S.A.), followed by incubation with the proper horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 hr. The Enhanced Chemiluminescence kit (Pierce) was used to detect the bound antibody.

Immunohistochemistry —— After de-waxing, rehydration, and antigen retrieval, lung sections were incubated with either a Kv1.5 (rabbit polyclonal antibody, 1 : 100 dilution, Santa Cruz) or Kv2.1 (goat polyclonal antibody 1 : 100 dilution, Santa Cruz) antibody for 1 hr at 37°C. The slides were washed and incubated with the appropriate HRP-labeled secondary antibodies for 30 min. After washing, the slides were incubated with 3,3′-diaminobenzidine (DAB, Pierce) and observed under a microscope.

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Staining —— Paraffin-embedded lung specimens were cut at 5 μm. Staining was performed using an alkaline phosphatase (AP)-based in situ cell death detection kit (Roche Diagnostics GmbH, Basel, Switzerland) according to the manufacturer's instructions. Briefly, after deparaffini zation and rehydration, tissue sections were treated with 20 mg/ml proteinase K at room temperature for 30 min. Tissue sections were then washed with phosphate-buffered saline and incubated with fluorescein-labeled nucleotides and terminal deoxynucleotidyl transferase at 37°C for 60 min in the dark, after which they were washed and incubated with converter AP (anti-fluorescein antibody conjugated with the reporter enzyme AP) for 30 min at room temperature. The reaction was developed with Fast Red tablets in naphthol phosphate substrate (Laboratory Vision Corporation, Fremont, CA, U.S.A.).

Statistical Analyses —— Data are expressed as means ± standard error (S.E.). Inter-group differences were analyzed using the Student's *t*-test and factorial analysis of variance (ANOVA). Differences were considered as statistically significant when $p < 0.05$.

RESULTS

Body Weight and Food Intake

As shown in Table 1, the body weight of HA and HA+DCA rats increased very slowly during the first 7 days of HA exposure. Compared with the N rats, no significant difference was observed after 3 days of HA exposure, while a significant decrease was detected at 7 days in the HA group. After 7 days of adaptation in the hypoxic environment, body weight dramatically increased at 14 and 21 days but was still significantly less than that of the N rats. No significant differences were detected among the HA and HA+DCA groups at any time point measured. Among the different groups, food and water intake changed in a manner similar to that of body weight (data not shown).

Hemodynamics

As shown in Fig. 1A and 1C, The mean pulmonary artery pressure (mPAP) was increase with the time prolong, the mPAP was increased significantly in HA rats (38.34 ± 3.35 mmHg) compared to N rats (18.22 ± 1.41 mmHg, $p < 0.01$), and DCA remarkably decreased the mPAP (25.22 ± 2.43 mmHg) compared to the HA rats ($p <$

Table 1. Body Weight (BW) of Rats before HA or at Different Time Points after HA Treatment ($\overline{x} \pm$ S.E.)

Groups	before HA (g)	3 d (g)	7 d (g)	14 d (g)	21 d (g)
N (n = 8)	229 ± 12	241 ± 13	254 ± 12	275 ± 10	304 ± 14
HA (n = 8)	229 ± 12	231 ± 10	233 ± 10*	254 ± 9**	277 ± 9**
HA+DCA (n = 8)	228 ± 13	230 ± 12	231 ± 13*	256 ± 12*	280 ± 101**

*$p < 0.05$ versus N, **$p < 0.01$ versus N.

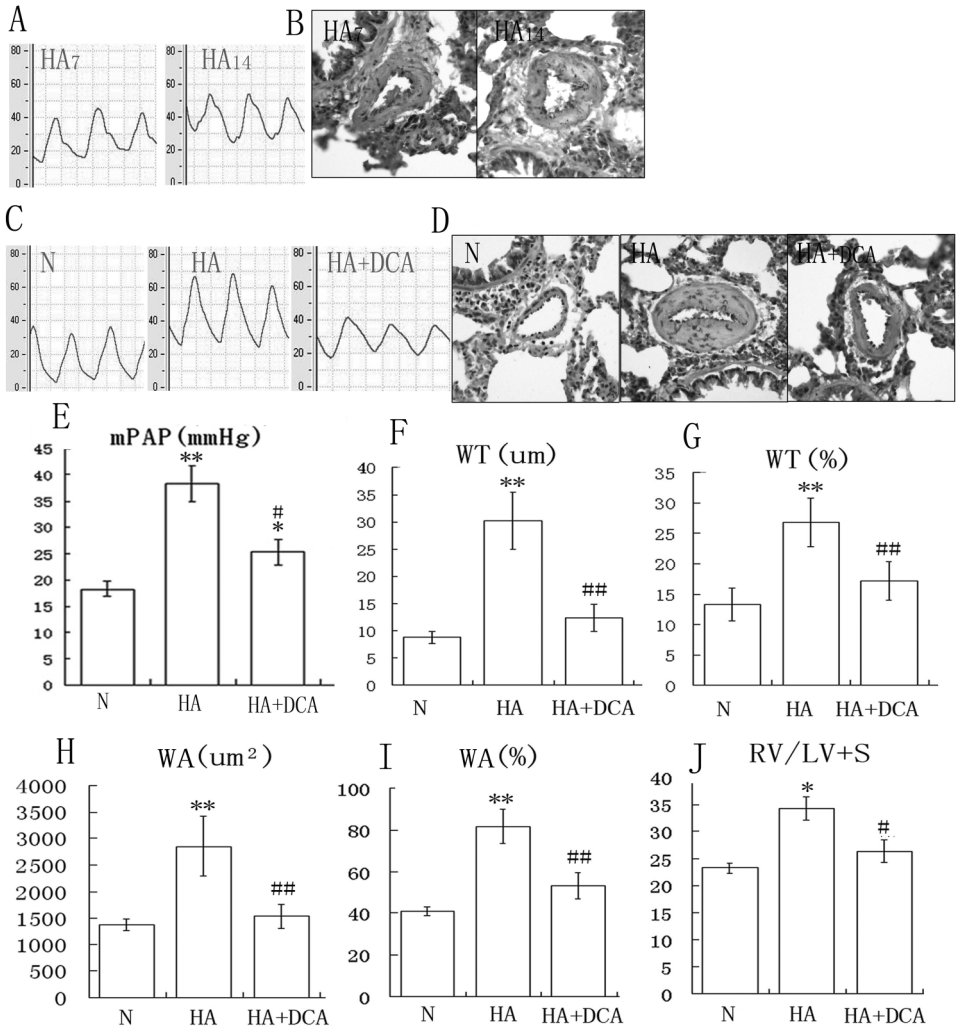

Fig. 1. DCA Improves Hemodynamics in HA-PAH

A and C, Representative high fidelity PA pressure (*$p < 0.05$, **$p < 0.01$ *vs.* N, #$p < 0.05$ *vs.* HA). B, the PAs thickness with the hypoxia; D, F–I, the percent medial thickness of resistance PAs is reduced by DCA treatment, WT, the percentage of WT/external diameter × 100% (WT%). WA was defined as the wall area of the small PAs and WA% is expressed as the percentage of wall area/whole small PAs area. E, mPAP: the mean pulmonary artery pressure. The mPAP reduced by DCA. J, RV/LV+S, the percent right ventricle to the left ventricle plus septum. DCA (70 mg/kg per day) prevented PA remodeling and RV hypertrophy (*$p < 0.05$, **$p < 0.01$ *vs.* N, #$p < 0.05$, ##$p < 0.01$ *vs.* HA). 30 PAs/group; 2–3 slides/rat, 3 rats/group.

0.01). There was no significant mean system blood pressure (mSBP) differences among these groups [mSBP (mmHg): N, 106 ± 9; HA, 112 ± 6 and HA+DCA, 109 ± 7]. RV hypertrophy (Fig. 1J) was increased significantly in the HA rats compared to the N rats but decreased in the HA+DCA rats compared to the HA rats.

PA and RV histology

The thickness of PAs in HA rats was notably increased followed with the time, with marked proliferation of PASMCs (shown in Fig. 1B and 1D);

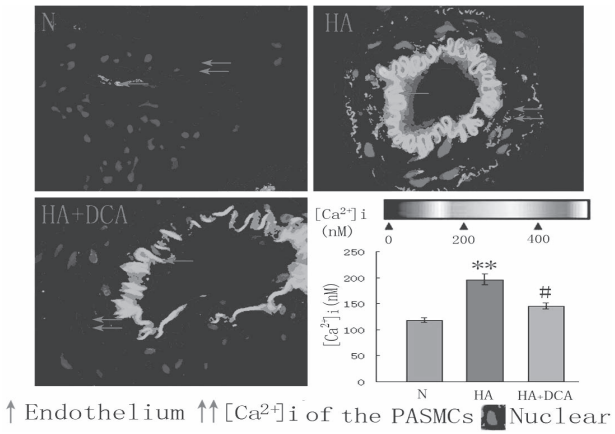

Fig. 2. Fluo-3-AM Stains the Free Ca^{2+} in the SMCs of the Small PAs

Ca^{2+} staining was much stronger in the HA group than in the N group ($^{**}p < 0.01$) and was weaker in the DCA+HA group compared to the HA group ($^{\#}p < 0.05$, × 600).

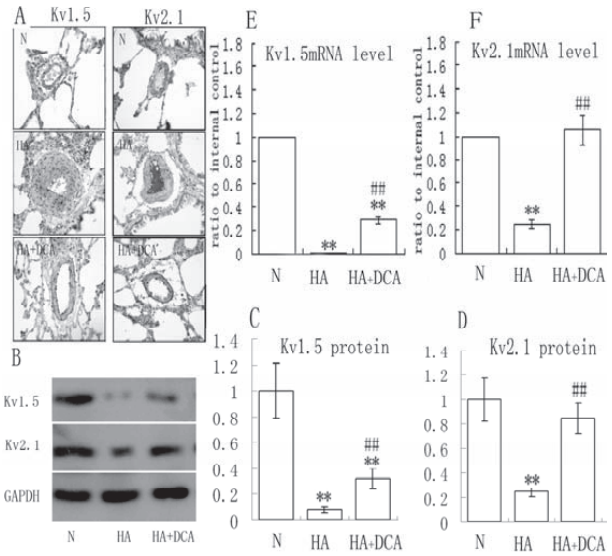

Fig. 3. DCA Improve the Kv1.5 and Kv2.1 Gene Expression

Immunohistochemistry (A) and Western blotting (B–D) for measurement of Kv1.5 and Kv2.1 protein expression in PASMCs ($^{**}p < 0.01$ *vs.* N, $^{\#\#}p < 0.01$ *vs.* HA). Real-time PCR (E–F) evaluation the Kv1.5 and Kv2.1 mRNA expression ($^{**}p < 0.01$ *vs.* N, $^{\#\#}p < 0.01$ *vs.* HA). Kv1.5 and Kv2.1 protein expression was assayed by immunohistochemistry. Cell nuclei were lightly stained with hematoxylin ($^{**}p < 0.01$ *vs.* N, $^{\#\#}p < 0.01$ *vs.* HA).

in response to DCA treatment, the PA thickness and proliferation of PASMCs were alleviated. WT, WT%, RV/LV+S, WA and WA% were significantly increased in the HA rats compared with those values of the N rats (Fig. 1F–1J); however, with DCA treatment, these data improved.

Effect of DCA on the $[Ca^{2+}]i$ of PASMCs in HA Rats

As shown in Fig. 2, the $[Ca^{2+}]i$ signal was very weak in the PASMCs of N rats, but it was very strong in HA rats. DCA markedly decreased the $[Ca^{2+}]i$ of PASMCs compared with that observed in the HA rats, although it was still greater than that in the N rats.

mRNA Expression of Kv1.5 and Kv2.1

As shown in Fig. 3E and 3F, Kv1.5 and Kv2.1 mRNA expression was significantly downregulated in HA PASMCs compared to N PASMCs, and DCA partially reversed the downregulation of Kv1.5 and nearly restored normal Kv2.1 expression.

Effects of DCA on Kv1.5 and Kv2.1 Protein Expression in HA Rats

As shown in Fig. 3A–3D, we carried out immunoblotting to examine the protein expression of Kv1.5 and Kv2.1. The protein expression of Kv1.5 and Kv2.1 in the PASMCs of HA rats was decreased compared to the expression in the N group, and the proliferation of PASMCs markedly increased in the HA group, resulting in lumenal stenosis. The Kv1.5 and Kv2.1 protein expression was restored, and the proliferation of PASMCs and lumen stenosis were attenuated in the DCA-treated group.

Western blotting demonstrated that the protein expression of Kv1.5 and Kv2.1 in the PASMCs of the HA group was markedly decreased compared to that of the N group. The expression of Kv1.5 was partially restored, and Kv2.1 expression was nearly restored to normal levels in the DCA-treated groups (Fig. 3B–3D).

PASMC Apoptosis

As shown in Fig. 4, TUNEL-positive cells indicate apoptotic nuclei. Compared with the N group, the apoptosis rate of the HA group were not significantly different, but compared with the HA group, the apoptosis rate of the DCA-treated rats was increased significantly.

DISCUSSION

In the present study, rats exposed to conditions simulating an altitude of 5000 m for 21 days displayed a significant increase in mPAP and simultaneously developed RV hypertrophy and pulmonary artery remodeling, simulating a high-altitude pulmonary artery hypertension rat model. The increases in mPAP and RV hypertrophy in the HA rats

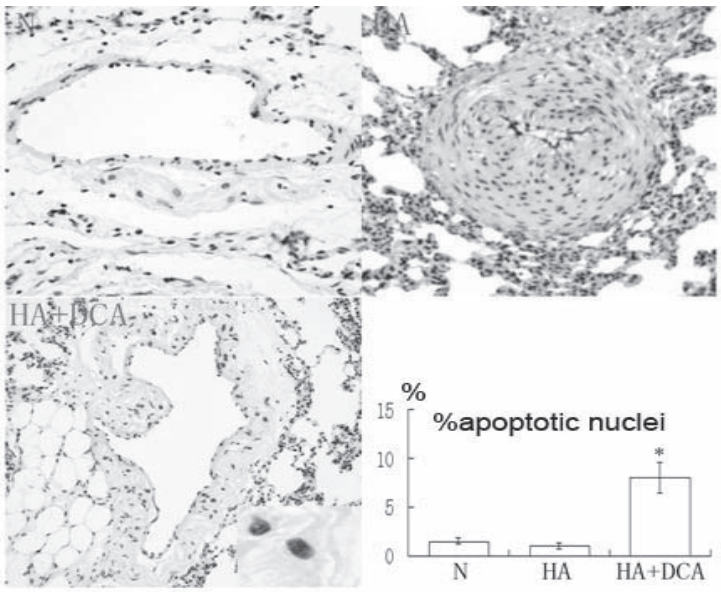

Fig. 4. DCA Reverses PA Remodeling by Increasing Apoptosis in the Media
There are normally few apoptotic cells, $^{*}p < 0.01$ *vs.* HA (2 slides/rat; 8 rats/group).

were almost entirely prevented by treatment with DCA. DCA also provided beneficial effects on the hemodynamic changes and PA remodeling. These results indicate that DCA can prevent and partially reverse HA-PAH in rats. DCA did not affect systemic pressure, left ventricle contraction, or heart rate, suggesting that DCA has a specific effect on pulmonary circulation.

Because K^+ channels are an important determinant in vascular tone and the proliferative status of vascular smooth muscle cells, the role of the K^+ channel and membrane potential has been investigated in several chronic pulmonary hypertension and acute hypoxia animal models.[12, 18, 21, 22] Previous studies have shown that chronic hypoxia decreases Kv gene expression.[1, 2, 10] Here, we showed that Kv1.5 and Kv2.1, the channels that participate in determining membrane potential in rat PASMCs, were downregulated in PASMCs during simulated HA. These data support the concept that Kv channel inhibition and downregulation may be etiologically related to the development of PAH.[23] In idiopathic pulmonary artery hypertension, a specific downregulation of Kv1.5 in PASMCs is associated with the development of, or at least a predisposition to, the disease.[24] Smirnov *et al.*[25] showed that the function of the Kv channel is inhibited in PASMCs from PAH rats and that PASMCs are depolarized. Many other studies have also shown that PAH is associated with decreased Kv1.5 and Kv2.1 expression.[24, 25] However, it remains unclear whether these changes in Kv function and expression are etiologically linked to HA-PAH. The reduction of Kv channel activity and the subsequent membrane depolarization appear to be involved in the development of chronic pulmonary hypertension by mediating pulmonary vasoconstriction and vascular remodeling through an increase of $[Ca^{2+}]i$,[3, 4, 26] which suggests that PA remodeling is correlated with $[Ca^{2+}]i$. We showed that the $[Ca^{2+}]i$ increased in the PAs of HA rats, which may be the mechanism underlying the specific Kv channel that mediates HA-PAH.

We demonstrated that DCA could up-regulate Kv1.5 and Kv2.1 expression and decrease $[Ca^{2+}]i$ in HA rats. Our data suggest that the effects of DCA on the function and expression of Kv channels are responsible for its ability to prevent and reverse HA-PAH. The downregulation of Kv1.5 and Kv2.1 under hypoxic conditions confirmed previously published literatures.[1, 11, 12] Rozanski *et al.*[26] showed that cultured cardiomyocytes from infarcted rat hearts exhibit an increase in delayed rectifier K^+ current (*Ik*) when incubated with DCA for 4 hr. This effect was mimicked by pyruvate and inhibited by a pyruvate dehydrogenase blocker, suggesting that the effects of DCA are due to metabolic changes. Evangelos *et al.*[27] showed that a low dose of DCA activates K^+ currents in chronic hypoxia PASMCs within 5 min, and this effect was

inhibited by 4-aminopyridine (4-AP), a specific Kv channel inhibitor. This finding indicated that DCA activates the Kv channel, but not the potassium-calcium (Kca) channel. The activity of certain Kv channel subtypes and Kca channels is sensitive to O_2,[1, 2, 11, 22] indicating that the effects of DCA involve a redox-dependent pathway.[28]

Muscle remodeling in PAH is due to an imbalance of proliferation and apoptosis.[16, 29] A recent study showed that the PA remodeling caused by elastase inhibitors is associated with an induction of apoptosis and a decrease in cell proliferation in the media of the PAs.[30] In our study, we also found that apoptosis was increased with the up-regulation of Kv1.5 and Kv2.1. Brevnona *et al.*[31] showed that overexpression of the Kv1.5 gene in PASMCs induces apoptosis. Kv2.1 gene transfer-induced apoptosis has also been observed in neurons and Chinese hamster ovary cells, which are deficient in functional Kv2.1-encoded K^+ channels.[32] DCA increases H_2O_2 production and reverses PA remodeling by inducing apoptosis and suppressing proliferation in monocrotaline-induced PAH.[16] H_2O_2 can activate redox- and 4-AP-sensitive Kv channels in coronary vascular smooth muscle.[29] Kv1.5 and Kv2.1 control the membrane potential in PASMCs.[3, 8] Activating and up-regulating these channels could explain the hemodynamic effects of DCA.

Meanwhile, downregulation of the Kv channel leads to depolarization of PASMCs, opening of voltage-gate Ca^{2+} channels, and increasing intracellular Ca^{2+}, which leads to vasoconstriction and increased PASMC proliferation.[3, 4, 33] Our study indicates that DCA could inhibit the proliferation of PASMCs by up-regulating Kv channels and reducing ($[Ca^{2+}]i$). DCA enhances apoptosis in monocrotaline-induced PAH by increasing H_2O_2 production.[16] In our study, the DCA-treated rats displayed decreased PA proliferation and increased PASMC apoptosis, which was supported by the fact that DCA can suppress remodeling in PASMCs.

Overall, DCA is capable of restoring Kv channel function and thus could be beneficial for treating HA-PAH. Importantly, DCA has already been used in small, short-term human studies without major toxicity.[34]

Acknowledgements We thank Professor Y. J. Shi, Lab of Transplant Engineering and Immunology, West China Hospital, Sichuan University, for his assistance. This study is supported by a grant from the Bureau of Health of Sichuan Province (090002) and was supported by the Science Grant of Chengdu Military General Hospital PLA (08Y05).

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