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Dichloroacetate, a Metabolic Modulator, Prevents and Reverses Chronic Hypoxic Pulmonary Hypertension in Rats
Role of Increased Expression and Activity of Voltage-Gated Potassium Channels

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Background—Chronic hypoxic pulmonary hypertension (CH-PHT) is associated with suppressed expression and function of voltage-gated K⁺ channels (Kv) in pulmonary artery (PA) smooth muscle cells (SMCs) and a shift in cellular redox balance toward a reduced state. We hypothesized that dichloroacetate (DCA), a metabolic modulator that can shift redox balance toward an oxidized state and increase Kv current in myocardial cells, would reverse CH-PHT.

Methods and Results—We studied 4 groups of rats: normoxic, normoxic + DCA (DCA 70 mg · kg⁻¹ · d⁻¹ PO), chronically hypoxic (CH), and CH + DCA. CH and CH + DCA rats were kept in a hypoxic chamber (10% Fio₂) for 2 to 3 weeks. DCA was given either at day 1 to prevent or at day 10 to reverse CH-PHT. We used micromanometer-tipped catheters and measured hemodynamics in closed-chest rats on days 14 to 18. CH + DCA rats had significantly reduced pulmonary vascular resistance, right ventricular hypertrophy, and PA remodeling compared with the CH rats. CH inhibited Iₖ, eliminated the acute hypoxia–sensitive Iₖ, and decreased Kv2.1 channel expression. In the short term, low-dose DCA (1 μmol/L) increased Iₖ in CH-PASMCs. In a mammalian expression system, DCA activated Kv2.1 by a tyrosine kinase–dependent mechanism. When given long-term, DCA partially restored Iₖ and Kv2.1 expression in PASMCs without altering right ventricular pyruvate dehydrogenase activity, suggesting that the beneficial effects of DCA occur by nonmetabolic mechanisms.

Conclusions—DCA both prevents and reverses CH-PHT by a mechanism involving restoration of expression and function of Kv channels. DCA has previously been used in humans and may potentially be a therapeutic agent for pulmonary hypertension. (Circulation. 2002;105:244–250.)

Key Words: hypertension, pulmonary ■ kinases ■ dichloroacetate ■ metabolism ■ ion channels

Exposure of animals or humans to chronic hypoxia (CH) leads to the development of chronic hypoxic pulmonary hypertension (CH-PHT) by an unknown mechanism. CH-PHT is characterized by pulmonary arterial (PA) vasoconstriction and remodeling.¹ Although the role of endothelium in the pathogenesis of CH-PHT is important, the role of vascular smooth muscle cells (SMCs) is increasingly recognized.² Both the contractile status and proliferative status of SMCs are regulated by the levels of the intracellular Ca²⁺ ([Ca²⁺]), levels are determined in part by influx of Ca²⁺ through the voltage-gated, L-type Ca²⁺ channels, the gating of which is controlled by the SMC membrane potential. In the PASMCs, the membrane potential is regulated by voltage-gated K⁺ channels (Kv), including Kv1.5 and Kv2.1.³ These channels, as well as Kv1.2, Kv3.1b, and Kv9.1, are O₂-sensitive and can be inhibited by hypoxia in expression systems (for review see Reference 3). Inhibition of one or more of these channels by acute hypoxia contributes to the initiation of hypoxic pulmonary vascular constriction.³ CH reduces K⁺ current density in PASMCs, resulting in a state of depolarization,⁴ which elevates [Ca²⁺], and thus promotes contraction and proliferation.⁵ CH is associated with impaired expression of certain Kv channels (eg, Kv1.5 and Kv2.1), although the expression of many other channels is unaltered.⁶⁷ The mechanism for Kv channel downregulation is unclear, but recent work suggests that this channel remodeling may relate to the altered redox state induced by CH.⁴ Lungs of rats with CH-PHT are in a more reduced redox state than those of normoxic controls, as indicated by a sustained reduction in activated oxygen species production and increased levels of reduced glutathione.⁴ A reduced redox state has potential for both short-term hemodynamic effects (through modulation of K⁺ channel function)⁸ and long-term effects (because several oxygen-responsive genes, including hypoxia-inducible factor, are redox regulated)⁹.
We hypothesized that downregulation of Kv channel function and expression is causally related to the development and maintenance of CH-PHT. We sought to enhance expression and function of Kv channels using dichloroacetate (DCA), a metabolic modulator, which has been shown to increase whole-cell K⁺ current (I_{K}) in cardiac myocytes from a rat myocardial infarction model.¹⁰ DCA inhibits mitochondrial pyruvate dehydrogenase kinase (PDK)¹¹ and, by increasing the pyruvate/lactate ratio, might promote an oxidized state in PASMCs.¹² Thus, we speculated that DCA would reverse the reduced redox state in the PASMCs of CH-PHT rats and thereby enhance the activity and expression of Kv channels and reverse CH-PHT, mimicking the benefits of a return to normoxia.

**Methods**

Age- and weight-matched male Sprague-Dawley rats were randomized to the following groups: normoxia (n=10), normoxia+DCA (n=5 for CH and n=10). The CH and CH-DCA groups were kept in a normobaric hypoxic chamber (Reming Bioinstruments). The inspired oxygen fraction was decreased from 20% to 10% over 5 days and sustained at this level. The water for the CH-DCA group contained DCA 0.75 g/L, pH 7.0 (Aldrich), for an estimated daily dose of 70 mg · kg⁻¹ · d⁻¹. This dose is based on previous human studies.¹³⁻¹⁵ Exposure to DCA started either on day 1 (n=5) to test for prevention of CH-PHT or on day 10 (n=5) to test for reversal of CH-PHT.

**Hemodynamic Measurements**

Beginning on day 14, the rats were anesthetized (sodium pentobarbital, 50 mg/kg IP) and ventilated with room air, and systemic blood pressure (SBP) was recorded, as previously described.¹⁶ PA pressures were measured with a 1.4F catheter (Millar Instruments) delivered through the right jugular vein. The mean PA pressure (PAP) was determined by electronic averaging over 1 minute. Pulmonary vascular resistance (PVR) was calculated as PAP–LVEDP/CO. The RV/(LV+S-epsilon) ratio, a measure of right ventricular hypertrophy (RVH), was measured as previously described.¹⁸

**Histology**

A section of lung was formalin-fixed for histology studies. Medial thickness was measured in small and medium-size PAs (n=88 from 4 rats per group) by investigators blinded to the treatment groups, as previously described.¹⁸

**Electrophysiology**

Fresh PASMCs were isolated from third to sixth division PAs and studied by use of the amphotericin-perforated patch-clamp technique, as previously described.¹⁹,²⁰ The pH of both 4-aminopyridine (4-AP) and DCA were adjusted to 7.4 before administration. Cells were voltage-clamped at a holding potential of −70 mV, and currents were evoked by steps from −70 to +50 mV with test pulses of 200 ms. Whole-cell currents were divided by the capacitance of the cell, giving a measure of current density.

Chinese hamster ovary (CHO) cells expressing green fluorescent protein (GFP) and Kv2.1 were also studied with conventional whole-cell technique. A recombinant, replication-deficient adenovirus encoding GFP and Kv2.1 was prepared by use of Adeasy-1, an adenoviral backbone vector that contains adenovirus (serotype 5) genomic DNA with E1 and E3 deleted. A 2609-bp cDNA fragment encoding the open reading frame of the rat Kv2.1 channel was excised from its original pBK-CMV plasmid (provided by Dr K. Takimoto, University of Pittsburgh, Pittsburgh, Pa) with restriction endonucleases NotI and SalI and ligated into pAdTrack-CMV, which contains a kanamycin resistance gene and 2 cytomegalovirus (CMV) promoters located upstream of both GFP and Kv2.1. When pAdTrack-CMV and Adeasy-1 are cotransformed into BJ5183 cells, the Kv2.1 gene undergoes homologous recombination with the adenoviral backbone, resulting in a plasmid that contains the Kv2.1 and GFP genes. The resultant pAdTrack-CMV Kv2.1 construct was linearized with a PmeI restriction endonuclease digest, transformed together with supercoiled adenoviral vector Adeasy-1 into BJ5183 cells and plated on LB plates containing kanamycin. Subsequent colonies were isolated, and the plasmid DNA was purified with plasmid purifying columns (Qiagen). The plasmid containing Kv2.1 cDNA within the adenoviral DNA was selected, amplified, purified, linearized, and transfected into HEK 293 cells with LipfectAmine reagent. Plates that demonstrated complete cell lysis were collected and analyzed for Kv2.1 by polymerase chain reaction. Multiple rounds of Ad5Kv2.1 replication were performed in HEK 293 cells. The resulting virus was isolated, precipitated, and concentrated by discontinuous CsCl gradient. The final viral titer was 1.5×10⁹ pfu/mL. Incubation for 24 hours with Ad5-GFP+Kv2.1 achieved 90% infection rates in CHO cells grown in hypoxia. Infected cells were selected on the basis of their green fluorescence (excitation 488 nm, detection 505 to 530 nm, with the LSM-510 confocal microscope by Zeiss), and the effects of DCA on the Kv2.1 current were studied.

Immunoblotting was performed on pooled PA samples (n=3 rats per group; 25 μg protein per group with antibodies to Kv channels [Alomone] and isoform 1 of the PDK [Stressgen Biotechnologies]), as previously described.¹⁹

PDH activity was measured in the RVs from 5 rats per group with a radiometric assay as previously described.²¹

**Statistics**

Values are expressed as mean±SEM. Intergroup differences were assessed by a repeated-measures ANOVA or factorial ANOVA, as appropriate. Post hoc analysis used a Fisher’s protected least significant differences test (Statview 5.0, SAS Institute). A value of P<0.05 was considered statistically significant.

**Results**

**Hemodynamics and Histology**

CH increased PAP and PVR and caused RVH and PA medial hypertrophy (Figures 1 to 3). Figures 1 to 3 show data from the first set of experiments, in which DCA was given in the CH-DCA rats on day 10 of the protocol to test for regression of CH-PHT. PAP and PVR were decreased in CH-DCA rats to values only slightly higher than in normoxic rats (Figures 1 and 2). The CO (Figure 2), SBP, and LVEDP were not different between the groups (SBP [mm Hg]: normoxic, 102±6; CH, 110±5; CH-DCA, 100±7; and LVEDP, 9 to 10 mm Hg for all groups). RVH was also significantly decreased in the CH-DCA compared with the CH rats (Figure 2).

In additional experiments, aimed at preventing CH-PHT, DCA was given on day 1 in the hypoxic chamber. In this protocol, an additional 3 groups of rats were used (normoxic, CH, and CH+DCA, n=5 for each). On days 14 to 18, PVR...
was significantly reduced in CH-DCA rats: normoxia, 0.09 ± 0.01; CH, 0.20 ± 0.02; and CH-DCA, 0.12 ± 0.02 mm Hg · min⁻¹ · mL⁻¹, \( P < 0.05 \). The CO, SBP, and RVH measurements were similar to those in the regression protocol (not shown). In a different experiment, DCA was given for 12 to 15 days in normoxic control rats \( (n=5) \) and had no effects on SBP or pulmonary hemodynamics (PVR, 0.10 ± 0.03 mm Hg · min⁻¹ · mL⁻¹).

**PASMC Electrophysiology**

\( I_K \) and current density were significantly decreased in CH rats and were largely restored in CH-DCA rats (Figure 4). As expected, the PASMC capacitance, which reflects cell size, was increased in the CH-PHT group \( (16±0.7 \text{ pF}) \) compared with normoxic controls \( (12.7±0.6 \text{ pF}) \), and this was reversed in the CH-DCA group \( (13.5±0.4 \text{ pF}) \). In normoxic control rats, 50% of \( I_K \) was inhibited by 4-AP \( (5 \text{ mmol/L}) \), suggesting that it is carried by Kv channels, whereas \( I_K \) in CH rats was insensitive to 4-AP (Figure 5A). In the CH-DCA rats, sensitivity to 4-AP was restored.

Acute hypoxia significantly inhibited \( I_K \) in normoxic but not CH PASMCs. There was a strong trend toward recovery of the \( O_2 \)-sensitive component of \( I_K \) in CH-DCA PASMCs \( (P<0.06) \) (Figure 5B). Short-term administration of low-dose DCA \( (1 \mu \text{mol/L}) \) increased \( I_K \) in CH PASMCs within 5 minutes of application but had no effect on \( I_K \) in normoxic or CH-DCA rats (Figure 5C).

**Immunoblotting**

Although Kv1.5 and Kv2.1 were significantly downregulated in CH PAs, the expression of other Kv channels and the large-conductance calcium-sensitive K⁺ channels \( (\text{BK}_{\text{Ca}}) \) did not change significantly (Figure 6). Although treatment with DCA did not affect the expression of Kv1.5, it partially reversed the downregulation of Kv2.1 (Figure 6). Figure 6 also shows that although the expression of PDK (isoform 1) appears to be somewhat increased in the CH rats compared with the normoxic rats, it did not change further with DCA.

**CHO Cell Electrophysiology**

Uninfected CHO cells had no Kv current (Figure 7). The successfully infected CHO cells were selected by their green fluorescence, and they were found to have an early activating, noninactivating, and voltage-dependent current that was significantly inhibited by low-dose 4-AP \( (1 \text{ mmol/L}) \), ie, a Kv2.1 current (Figure 7). DCA had no effects on the control CHO cells. In contrast, 1 µmol/L DCA rapidly activated the Kv2.1 current in the infected CHO cells. Pretreatment with the tyrosine kinase inhibitor genistein \( (100 \mu \text{mol/L}, \text{Sigma}) \) prevented effects of DCA on \( I_K \) without inhibiting the basal Kv2.1 current (Figure 7).

**PDH Activity**

The activity of PDH is directly regulated through phosphorylation by the PDK. We studied the RV because the amount of PA tissue was insufficient. The total PDH activity as well as the active/total PDH activity, at least in the RV, was not different among the 3 groups: active/total normoxic, 0.72±0.11; CH, 0.63±0.12; CH-DCA, 0.79±0.13; \( P=\text{NS} \).

**Figure 2.** Mean hemodynamic data from 3 rat groups studied in regression protocol. DCA reverses increase in PAP, PVR, and RVH caused by CH but has no significant effect on CO.

![Mean PAP](image1)

![PVR](image2)

![CO](image3)

![RV/LV+S](image4)
Discussion
We show that DCA both prevents and reverses established CH-PHT. The beneficial effects of DCA on the hemodynamics (Figure 2) and the PA remodeling (Figure 3) parallel its effects on Kv channel expression and function (Figures 5 to 7). In the short term, DCA increases $I_K$ in freshly isolated PASMCs from rats with CH-PHT and specifically activates Kv2.1 channels expressed in hypoxic CHO cells. When given long-term, DCA reverses the CH-induced downregulation of Kv channels, specifically Kv2.1 (Figure 6). We propose that the effects of DCA treatment on the function and expression of Kv channels are responsible for the ability of this drug to reverse CH-PHT. To the best of our knowledge, this is the first study using micromanometer-tipped catheters to measure PAP in the more physiological closed-chest rat model. Because the chest is closed, the pressures are higher than in

Figure 3. A, Histology (H-E stain) of medium-size PAs from 3 rat groups studied. Representative PA is shown for each group (arrows). B, DCA decreases PA medial hypertrophy caused by CH.

Figure 4. Left, Mean data of PA SMC $I_K$ density plotted against voltages studied under patch-clamp protocol. Long-term treatment with DCA causes reversal of decrease in current density induced by CH across whole membrane potential spectrum studied. Bottom, Representative raw trace of $I_K$ from each group studied. For cells shown, capacitance for control, CH-PHT, and CH-DCA PA SMCs was 12.8, 15.9, and 13.6 pF, respectively.
the open-chest models usually used. These catheters also avoid the artifacts due to limited frequency response and damping inherent to fluid-filled catheters.

The effects of DCA on the LV performance have been studied extensively, with conflicting results, but its effects on the pulmonary circulation have not previously been studied. Our finding that DCA does not affect the LVEDP or SBP suggests that its effects are specific to the pulmonary circulation.

The data support the concept that Kv channel inhibition and downregulation might be etiologically related to the development of CH-PHT. In human primary pulmonary hypertension, a specific downregulation of PA Kv1.5 is associated with the development of or at least a predisposition to the disease. Smirnov et al first showed that in PASMCs from rats with CH-PHT, the function of Kv channels is suppressed and PASMCs are depolarized. It was later shown that CH-PHT is associated with decreased expression of Kv1.2 and Kv1.5, and Kv2.1. Uncertainty remains, however, as to whether these changes in Kv function and expression are etiologically linked to the disease or are just secondary phenomena. Our finding that DCA reversed CH-PHT by restoring both the function and expression of Kv2.1 supports a causal role for Kv channel deficiency in the pathogenesis of this form of experimental PHT.

We show that DCA upregulates expression of Kv2.1 (Figure 6), a channel that participates in determining membrane potential in rat PASMCs. Although immunoblotting is only a semiquantitative method, the downregulation of Kv1.5 and Kv2.1 in CH (Figure 6) confirms previously published data. These are probably not nonspecific effects, because other channels are not affected (Figure 6). More importantly, however, the immunoblotting data are in agreement with our electrophysiology data. The fact that 4-AP sensitivity of the $I_K$ is significantly diminished in the CH rats (Figure 5A) is in agreement with the observed decrease in the expression of the 4-AP-sensitive channels Kv1.5 and Kv2.1.

The lack of significant effects of 1 mmol/L 4-AP on normoxic $I_K$ (Figure 5A), however, suggests that Kv1.5, which is very sensitive to 4-AP, might not be as great a contributor to the $I_K$ as Kv2.1. The partial recovery of 4-AP sensitivity (Figure 5A) in the CH-DCA PASMCs is probably the functional consequence of the partial recovery of the Kv2.1 expression. Furthermore, the loss of the sensitivity to acute hypoxia in the CH rats (Figure 5B) is most likely due, in part, to the loss of the oxygen-sensitive channels, such as Kv1.5 and Kv2.1. Similarly, the trend in CH-DCA PASMCs to regain their responsiveness to acute hypoxia, albeit not statistically significant ($P<0.06$ Figure 5B), suggests that the upregulated Kv2.1 expression is functionally relevant. The
current-density data and the voltage dependence of the $I_K$ in PASMCs in this report are within the range published in the literature. Variability in these values can be explained in part by differences in the enzymatic dispersion techniques and the specific vascular sections used, because there is regional diversity in the expression of $K^+$ channels within the pulmonary circulation.

In addition to the long-term effects of DCA, we show that this drug can increase $I_K$ in the short term much more rapidly and at much lower doses than previously described (μmol/L versus mmol/L), indicating novel and previously unrecognized properties of DCA. Rozanski et al.10 showed that cultured cardiomyocytes from infarcted rat hearts have increased $I_K$ when incubated with 1.5 mmol/L DCA for 4 hours.

Figure 6. Immunoblotting data on isolated PAs. Background-corrected intensities normalized to Ponceau-S values are shown below each gel. CH causes a decrease in expression of Kv1.5 and Kv2.1 without significant effects on other $K^+$ channels tested. Long-term treatment with DCA partially reverses downregulation of Kv2.1 and has no significant effects in expression of other $K^+$ channels and PDK1.

Figure 7. Whereas CHO cells have no baseline fluorescence and display only a voltage-insensitive outward current, CHO cells expressing GFP and Kv2.1 have a strong green fluorescence and a voltage- and 4-AP–sensitive current. This Kv2.1 current is activated by 1 μmol/L DCA. Pretreatment with genistein prevents effects of DCA.
This was mimicked by pyruvate and inhibited by a PDH blocker, suggesting that the effects of DCA were due to the metabolic actions of the drug (eg, PDH activation).10 We now show that DCA activates K+ current in CH PASMCs within 5 minutes and at the very low dose of 1 μmol/L (Figure 5C). These observations are consistent with DCA acting through other kinases that may activate Kv currents.29 Because the Ik in vascular SMCs is an ensemble of multiple K+ channels, it is impossible to determine the molecular target of DCA in PASMCs. Therefore, we used a CHO expression system and showed that DCA specifically activates Kv2.1 by a mechanism involving tyrosine kinase (Figure 7). Because Kv2.1 controls membrane potential in PASMCs, activation and upregulation of this channel could explain the hemodynamic effects of DCA.

Although we showed that DCA, at least in the dose used, did not have any effect on PA PDK-1 expression (Figure 6) and RV PDH activity (Figure 7), we cannot exclude redox/metabolic effects on the PASMCs. PDK diversity and tissue-specific regulation of PDH are well described.30,31 In addition, vascular SMC energetics are understudied, and thus, it is unknown whether they parallel those of the heart.12 Our electrophysiology data, however, provide a novel mechanism that could operate in parallel to a redox mechanism. We suggest that DCA opens Kv2.1 via a tyrosine kinase–dependent mechanism and that this causes membrane hyperpolarization, and a decrease in [Ca2+]i. These actions promote vasorelaxation and inhibition of PASMС proliferation.

DCA is a very attractive drug to be studied in human PHT, particularly because it has already been used in small, short-term human studies without major toxicity.13–15 To the best of our knowledge, no other drugs in current clinical use have Kv channel opening properties. DCA may be capable of restoring K+ channel function and expression and thus have benefit in the treatment of pulmonary vascular diseases.

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