

Dichloroacetate Prevents and Reverses Pulmonary Hypertension by Inducing Pulmonary Artery Smooth Muscle Cell Apoptosis

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Abstract—The pulmonary arteries (PA) in pulmonary arterial hypertension (PAH) are constricted and remodeled; they have suppressed apoptosis, partly attributable to suppression of the bone morphogenetic protein axis and selective downregulation of PA smooth muscle cell (PASMC) voltage-gated K^+ channels, including Kv1.5. The Kv downregulation-induced increase in $[K^+]_i$, tonically inhibits caspases, further suppressing apoptosis. Mitochondria control apoptosis and produce activated oxygen species like H_2O_2 , which regulate vascular tone by activating K^+ channels, but their role in PAH is unknown. We show that dichloroacetate (DCA), a metabolic modulator that increases mitochondrial oxidative phosphorylation, prevents and reverses established monocrotaline-induced PAH (MCT-PAH), significantly improving mortality. Compared with MCT-PAH, DCA-treated rats (80 mg/kg per day in drinking water on day 14 after MCT, studied on day 21) have decreased pulmonary, but not systemic, vascular resistance (63% decrease, $P < 0.002$), PA medial thickness (28% decrease, $P < 0.0001$), and right ventricular hypertrophy (34% decrease, $P < 0.001$). DCA is similarly effective when given at day 1 or day 21 after MCT (studied day 28) but has no effect on normal rats. DCA depolarizes MCT-PAH PASMC mitochondria and causes release of H_2O_2 and cytochrome c, inducing a 10-fold increase in apoptosis within the PA media (TUNEL and caspase 3 activity) and decreasing proliferation (proliferating-cell nuclear antigen and BrdU assays). Immunoblots, immunohistochemistry, laser-captured microdissection-quantitative reverse-transcription polymerase chain reaction and patch-clamping show that DCA reverses the Kv1.5 downregulation in resistance PAs. In summary, DCA reverses PA remodeling by increasing the mitochondria-dependent apoptosis/proliferation ratio and upregulating Kv1.5 in the media. We identify mitochondria-dependent apoptosis as a potential target for therapy and DCA as an effective and selective treatment for PAH. (*Circ Res.* 2004;95:830-840.)

Key Words: apoptosis ■ proliferation ■ smooth muscle ■ vascular remodeling

Pulmonary arterial hypertension (PAH) is defined by an elevated pulmonary vascular resistance (PVR), which leads to right heart failure and premature death. The cause remains unknown and available treatments are limited, expensive, and often associated with significant side effects.^{1,2} The pulmonary arteries (PAs) are affected by varying degrees of vasoconstriction and vascular remodeling, including cellular proliferation in both the intima and media and distal PA muscularization.^{1,2}

Vascular medial remodeling results from an imbalance between smooth muscle cell (SMC) proliferation and apoptosis, favoring proliferation. Gene microarray studies show that lungs from patients with PAH have a decrease in the proapoptotic/antiapoptotic gene expression ratio.³ Furthermore, several loss-of-function germline or acquired mutations have been described in receptors of the transforming growth

factor- β superfamily, such as bone morphogenetic protein receptor-2 (BMPR-2), in patients with primary PAH.² Activation of the transforming growth factor- β /BMPR2 axis suppresses proliferation and activates apoptosis in normal PA smooth muscle cell (PASMC)⁴ but not in PASMC from patients with PAH.⁵ This resistance to apoptosis is further enhanced by the selective downregulation of Kv channels that has been shown in human^{3,6} and animal models of PAH.⁷ Intracellular K^+ levels, which increase when K^+ channels are inhibited or downregulated, exhibit a tonic inhibition of caspases in many cell types, including PASMC.⁸ In addition to a suppression in apoptosis, Kv channel downregulation leads to PASMC depolarization, opening of the voltage-gated Ca^{++} channels, and increased intracellular Ca^{++} ; in turn, this causes vasoconstriction and increased PASMC proliferation.⁹

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The role of mitochondria in pulmonary vascular biology and PAH is unknown. However, mitochondria are potentially important because they regulate both apoptosis (by the release of proapoptotic factors, including the caspase activator cytochrome c^{10}) and vascular tone (by the production of activated oxygen species [AOS]). AOS can “leak” to the cytoplasm and affect redox-sensitive second messenger systems and membrane K^+ channels. For example, superoxide is produced in the proximal electron transport chain (ETC) and, in the presence of the mitochondria-based manganese superoxide dismutase (MnSOD), it is dismutated to H_2O_2 , a well-characterized K^+ channel opener and vasodilator.^{11,12} Therefore, vascular mitochondria might be important targets for the treatment of vascular disease.

We show that the orally available metabolic modulator dichloroacetate (DCA), which enhances oxidative phosphorylation by inhibiting the mitochondrial pyruvate dehydrogenase kinase and has been used extensively in humans for mitochondrial diseases and lactic acidosis,¹³ prevents and reverses monocrotaline-induced PAH (MCT-PAH) in rats. DCA inhibits MCT-PAH by inducing mitochondria-dependent apoptosis and reversing the downregulation of Kv1.5 in the media of resistance PAs.

Materials and Methods

Experimental Protocols

Experiments were in accordance with the University of Alberta Animal Policy and Welfare Committee. MCT-PAH was induced by injection of MCT 60 mg/kg subcutaneously on day 1. Treated rats (MCT+DCA) were fed with DCA (0.75 g/L, pH 7.0; Sigma-Aldrich) in drinking water, with an average ingestion of 80 mg/kg per day,⁷ unless stated otherwise (see dose-response experiments). A total of 20 normal control rats (15 with no treatment and 5 with DCA treatment) were used. In the protocols listed, all rats were hemodynamically studied and tissues obtained for additional studies.

DCA was started on day 1. Treated (n=20) and nontreated MCT-PAH (n=10) rats were studied on day 21. For early reversal, DCA was started on day 14. Treated (n=10) and nontreated MCT-PAH (n=10) rats were studied on day 21. For late reversal, DCA was started on day 21. Treated (n=5) and nontreated MCT-PAH (n=9) rats were studied on day 28.

In additional time-course experiments, MCT-PAH rats were studied on days 0, 7, 14, 21, 28 (n=3 for each) and in parallel with additional MCT-DCA day 14 (n=5) and MCT-DCA day 21 (n=5) rats. Three additional MCT-PAH rats were followed-up from day 0 to day 28 with implanted telemetry catheters.

For dose-reponse studies MCT-PAH rats (n=10) were compared with rats treated with the following doses of DCA (g/L): 0.075 (n=3), 0.75 (n=4), and 7.5 (n=3), using the early reversal protocol. The rats included in all the protocols (MCT-PAH, n=51; MCT-DCA, n=55) were prospectively followed-up for survival analysis. Spontaneous deaths were counted as events. Rats undergoing planned hemodynamic evaluation and euthanization were censored. Time to event data were plotted using the Kaplan-Meier method, with differences evaluated using the log rank test.

Echocardiology

We used a SONOS 5500 machine with 15-Mhz and 12-Mhz probes (Philips Medical Systems). Right ventricular (RV) free-wall thickness, measured by M-mode, and PA Doppler signals were measured in parasternal short axis.¹⁴

Hemodynamics

Rats anesthetized with pentobarbital (50 mg/kg intraperitoneally) were orotracheally intubated, ventilated ($FiO_2=0.4$), and left heart (left ventricular end-diastolic pressure), carotid artery (arterial pressure), and PA catheterization were performed as described^{7,15} using 1.4-French Millar catheters (Millar Instruments). Cardiac index was measured using the Fick and thermodilution methods as previously described.^{7,15} Pulmonary vascular resistance index (PVRI) was calculated as (mean PA – left ventricular end-diastolic pressure)/cardiac index. For details on the telemetry recordings see the online supplement available at <http://circres.ahajournals.org>.

Morphometry, Immunoblotting, Immunohistochemistry, and Quantitative RT-PCR

For morphometric analysis, RV hypertrophy was measured as RV/(left ventricular+septum) weight and PA remodeling was measured as percent medial thickness^{7,15} (see online supplement).

Immunoblotting was performed on pooled PA samples (each band has PAs pooled from 3 rats/group; 25 μ g protein/pooled sample), as described^{7,11,15} (see online supplement). Paraffin-embedded lungs were sectioned and stained with antibodies to either Kv1.5 or Kv2.1 and counterstained with eosin, as described^{15,16}. Laser capture microdissection and quantitative RT-PCR were performed as previously described^{15,17,18} (see online supplement).

Electrophysiology

Fresh PASM were isolated from fourth to sixth division PAs and studied with whole-cell patch-clamping, as described^{7,11,15} (see online supplement). PASM isolation and culture data are available in the online supplement.

Confocal Microscopy

Imaging was performed using a Zeiss LSM 510 confocal microscope as described.^{7,15,17} Apoptag apoptosis detection kit (TUNEL stain; Serologicals, Norcross, Ga) and the proliferating cell nuclear antigen (PCNA) antibody (DAKO, Carpinteria, Calif) were used as per manufacturer's instructions on paraffin-embedded tissue sections after antigen retrieval (microwave). Counterstain with 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI, 300 nM; Molecular Probes) was performed for 10 minutes at 20°C and washed with phosphate-buffered saline. ApoAlert Annexin V kit (Clontech, Palo Alto, Calif) and cytochrome c antibody (Pharmingen, San Diego, Calif) were used as per manufacturer's instructions on cells exposed to either propidium iodide (500 nM; Molecular Probes, Eugene, Ore) or DAPI.

BrdU was injected (100 mg/kg intraperitoneally) 4 hours before euthanization, and lungs were formalin-fixed and paraffin-embedded. Staining of sections was performed as per manufacturer's instructions (Hoffman-La Roche).

Dichlorofluorescein Assay

For dichlorofluorescein assay, PAs were incubated in Krebs buffer supplemented with 10 μ mol/L dichlorofluorescein (Molecular Probes), and H_2O_2 production was measured as described¹¹ (see online supplement).

Statistics

Values are expressed as the mean \pm SEM. Intergroup differences were assessed by Kruskal-Wallis or 1-way ANOVA as appropriate with post hoc analysis using Fisher exact test (Statview 4.02; SAS Institute, Cary, NC).

Results

DCA Therapy Improves Survival Without Evidence of Toxicity

We studied the rats within 4 weeks after MCT injection to prevent the animals from experiencing the severe heart failure

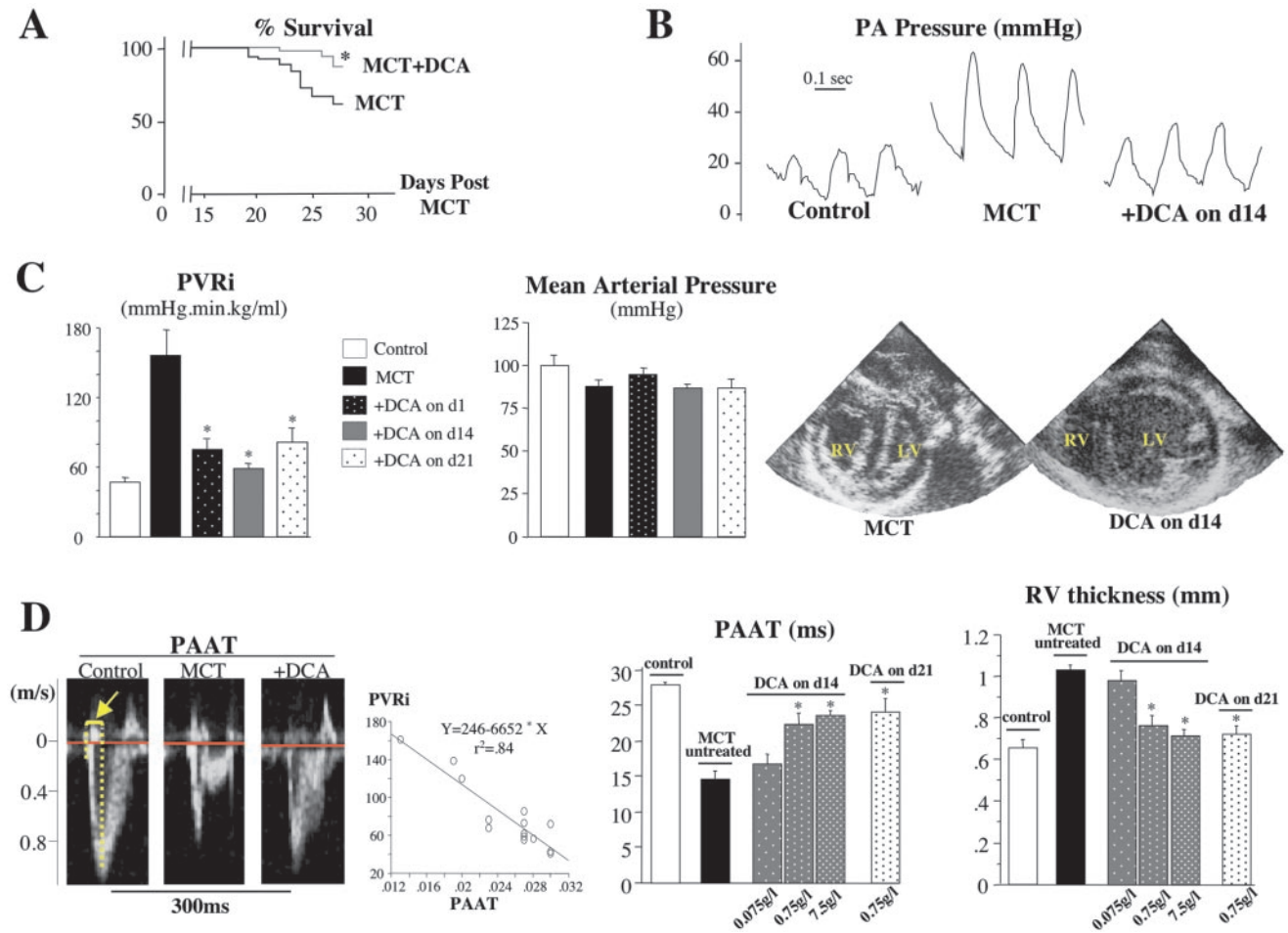


Figure 1. DCA improves hemodynamics in MCT-PAH. A, Kaplan-Meier curve showing a significant survival benefit in the DCA-treated rats compared with the untreated MCT-PAH rats (n=55 and 51, respectively). Representative high-fidelity PA pressure tracings (B) and mean PVRi and systemic blood pressure data (C). Representative echocardiograms demonstrating septal shift in MCT-PAH, reversed by DCA treatment. D, Regression analysis shows that PAAT predicts invasively measured PVRi. ECHO data on the early (3 different doses) and late reversal protocols show that DCA reverses the PAAT shortening and RVH caused by MCT (**P*<0.01 versus MCT).

and morbidity associated with the rapid worsening of PAH beyond 4 weeks.^{19,20} DCA significantly improves survival in MCT-PAH and the positive effect on survival is present 1 week after initiation of treatment (Figure 1A). Furthermore, the DCA-treated rats showed no evidence of liver, renal, or blood toxicity (see online Table I).

DCA Normalizes the PVRi in PAH Without Affecting Systemic Hemodynamics

DCA-treated rats had significantly lower PVRi compared with nontreated MCT-PAH rats (Figure 1B, 1C), whereas arterial pressure (Figure 1C) and left ventricular end-diastolic pressure were not different (Table I). The PVRi of the DCA-treated rats in both the prevention and reversal protocols was normalized, ie, it was not statistically different from normal controls. The significant hemodynamic improvement in the DCA-treated rats was also confirmed echocardiographically. The shift of the interventricular septum toward the left ventricle, an index of severe RV pressure overload, was normalized in all DCA-treated rats (Figure 1C).

Another clinically useful physiologic index of PAH is the PA acceleration time (PAAT), ie, the time from the beginning to the

peak of the velocity envelop during pulsed Doppler interrogation of the pulmonary valve¹⁴ (Figure 1D). We show a strong correlation between PAAT and PVRi in rats simultaneously studied with ECHO and catheterization (the higher the PA pressure, the shorter the PAAT) (Figure 1D). PAAT was significantly decreased in the MCT rats versus controls and was increased in rats that received DCA both on day 14 and day 21 (Figure 1D).

Most of our experiments were performed with 0.75g/L, because at this dose DCA has been used safely and effectively in humans with metabolic disorders.¹³ To determine whether there is a dose response, in some rats we also used a lower and a higher dose (Figure 1D). We showed that the low dose 0.075g/L is probably a threshold dose, having minimal effects on hemodynamics. The high 7.5-g/L dose had only minimal additional effects compared with 0.75 g/L. However, at the high 7.5-g/L dose, the water tends to have a moderate odor and bitterness and, although not significant, the rats consumed less water plus DCA.

As predicted, DCA also caused reversal of RV hypertrophy (RVH) measured both echocardiographically (Figure 1D) and by RV/left ventricular+septum weight ratio (Table I). In keeping with the PAAT dose response data, the effects of the

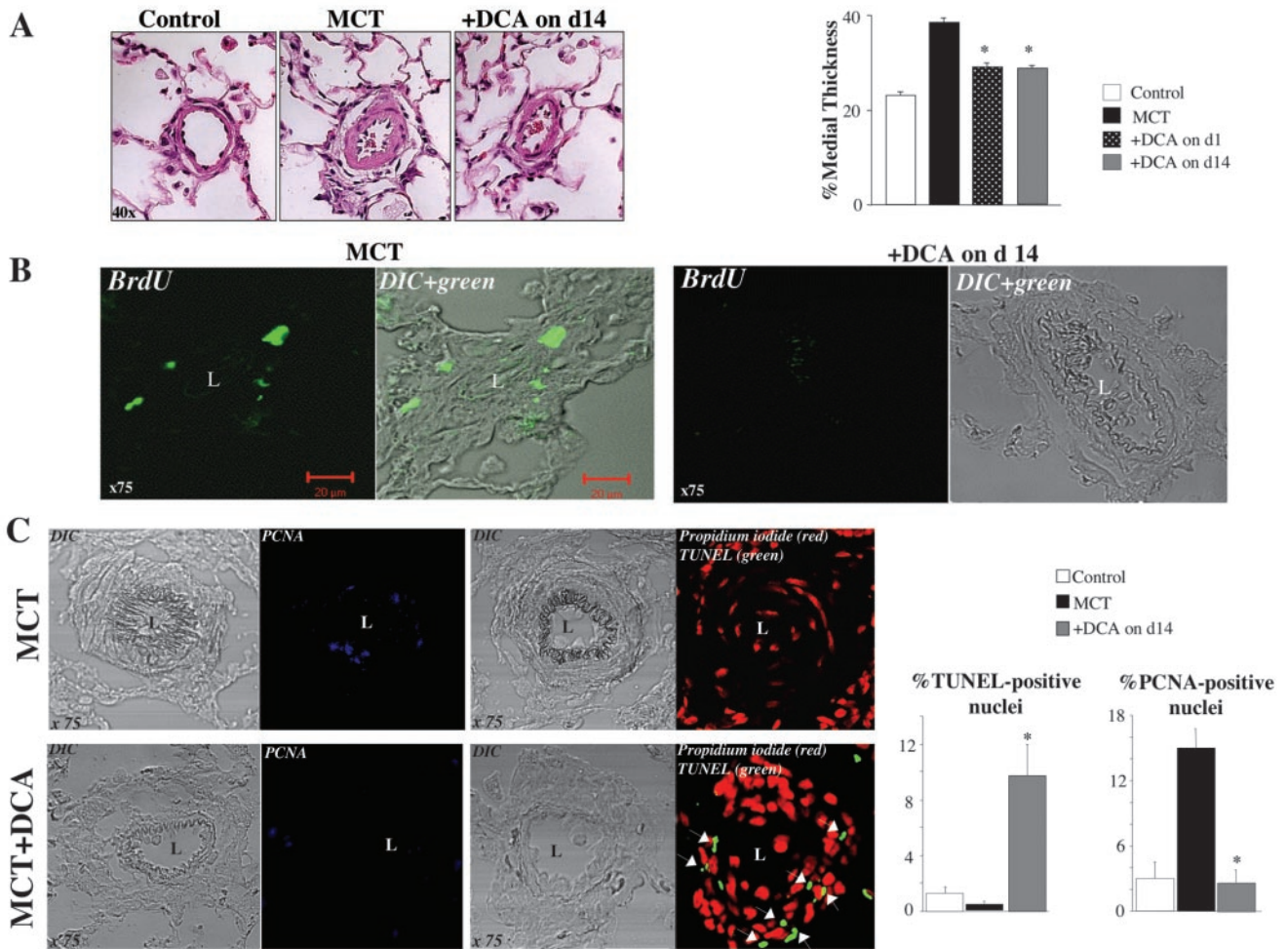


Figure 2. DCA reverses PA remodeling by increasing the apoptosis/proliferation ratio in the media. A, The percent medial thickness of resistance PAs is reduced by DCA-treatment ($*P < 0.05$ versus MCT, $n = 60$ PAs/group; 2 to 3 slides/rat, 3 rats/group). B and C, Representative images of MCT and MCT+DCA rat resistance PAs. In the MCT PAs, there is BrdU uptake and heavy expression of PCNA in the media, whereas there are no TUNEL-positive nuclei. In contrast, PAs from the DCA-treated rats have suppressed BrdU uptake and PCNA expression and a significant increase in the TUNEL-positive cells in the media. $*P < 0.001$ versus MCT, $n \approx 100$ PAs/group; 2 to 3 slides/rat; 5 rats/group. L indicates Lumen.

low dose DCA on RVH were minimal, whereas the two higher doses caused essentially a complete reversal of RVH (Figure 1D; online Figure I).

DCA Reverses PA Remodeling by Inducing Apoptosis and Suppressing Proliferation in the Media

As predicted by the hemodynamic data, DCA prevented and reversed the percent medial hypertrophy in MCT-PAH (Figure 2A). The media of the remodeled MCT-PAH PAs showed evidence of enhanced cell proliferation, with increased BrdU uptake (Figure 2B) and PCNA expression (Figure 2C), whereas in DCA-treated animals both BrdU uptake and PCNA expression were completely suppressed. Whereas the MCT-PAH PAs had no evidence of apoptosis (measured by TUNEL and propidium iodide-stained nuclear morphology), DCA-treated rats had a significant increase (≈ 10 -fold) in apoptosis (Figure 2C). In some MCT-PAH PAs, extensive PCNA expression and no apoptosis were seen in the absence of significant medial hypertrophy, suggesting that prolifera-

tion likely precedes, eventually leading to, PA remodeling (online Figure II). Essentially all the PCNA, BrdU, and TUNEL-positive nuclei were seen in the PA media and adventitia and not in the endothelium. Furthermore, DCA treatment caused activation of an important effector of apoptosis, caspase 3, in the PAs (Figure 3A).

DCA-Induced Apoptosis Is Mitochondria-Dependent, Accompanied by Increased H₂O₂ Production, and Occurs Early

Isolated PASMCS from MCT-PAH rats had a significant increase in mitochondria membrane potential ($\Delta\Psi_m$) versus control rats, and this increase was present even at 7 days after MCT injection, a time when the hemodynamic changes of PAH are not yet established. DCA treatment (early reversal protocol) decreased the $\Delta\Psi_m$ back to normal levels (Figure 3B).

Because AOS production increases when $\Delta\Psi_m$ depolarizes, and because the PASMCS mitochondria are enriched in manganese superoxide dismutase (at least compared with systemic vascular SMC¹¹), we studied H₂O₂ production from

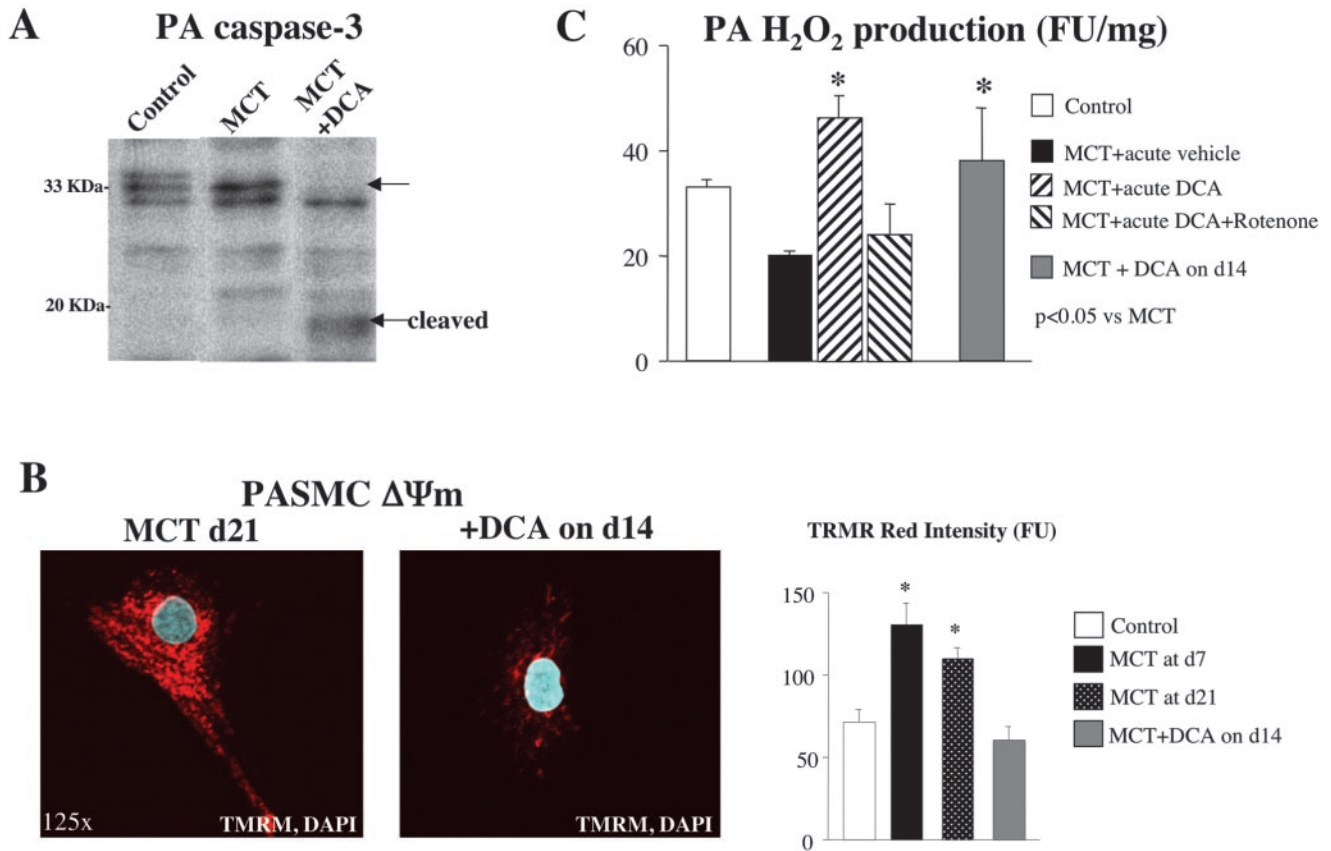


Figure 3. DCA increases H₂O₂ production, depolarizes mitochondria, and activates caspase 3 in MCT-PAH PAs in vivo. **A**, Caspase-3 in pooled PAs from MCT-DCA rats is cleaved (activated), compared with untreated MCT and control rats. **B**, DCA treatment normalizes the $\Delta\psi_m$ in PASMC, compared with PASMC from untreated MCT PAs ($n=20$ /group, cells from 3 rats/group). **C**, Chronic treatment with DCA normalizes the suppression of H₂O₂ production seen in MCT-PAH. Acute treatment with DCA increases H₂O₂ production and this is prevented by rotenone. * $P < 0.05$ versus MCT, $n=3$ /group.

freshly isolated PAs. Compared with controls, PAs isolated from MCT-PAH rats make less H₂O₂. The production of H₂O₂ increases to normal levels in the DCA-treated rats (Figure 3C). To determine whether DCA (100 μ mol/L) had any acute effects, we incubated the MCT-PAH PAs for 1 hour with DCA, and we showed that DCA increased the H₂O₂ production to the level of the rats chronically treated with DCA. We also showed that this acute increase was prevented by rotenone (5 μ mol/L, an inhibitor of the complex I of the mitochondrial ETC) (Figure 3C).

To determine whether DCA would have the same effects on mitochondria in vitro and in another model, we used chronically hypoxic PASMC (CH-PASMC) in primary culture. This model is relevant to PAH because chronic hypoxia is the most common cause of clinical PAH. Furthermore, we have recently shown that DCA reversed rat chronic hypoxia-induced PAH, but in that article we did not explore the effects of DCA on mitochondria and apoptosis pathways.⁷ DCA treatment (100 μ mol/L) caused CH-PASMC apoptosis measured by TUNEL and nuclear morphology (Figure 4A), which occurred as early as 6 hours, as shown by annexin staining (Figure 4B). DCA treatment caused leakage of the caspase activator cytochrome c from mitochondria (Figure 4C). DCA depolarized CH-PASMC mitochondria (Figure 4D). We also measured H₂O₂ production in freshly isolated

PAs placed in hypoxic tissue culture for 8 hours (online Figure III). Exposure to DCA (100 μ mol/L) increased and normalized H₂O₂ production in hypoxic PAs. DCA did not affect H₂O₂ production in normoxic PAs (not shown).

DCA Does Not Affect Normal PASMC and PAs

DCA does not affect the $\Delta\psi_m$ of freshly isolated PASMC from healthy rats and normal PASMC in primary culture (Figure 5A). Importantly, DCA does not induce apoptosis in normal PASMC (Figure 5B) and does not affect hemodynamics in healthy rats treated with DCA (Figure 5C) for the same duration and dose as the MCT-PAH rats.

DCA Treatment Reverses the Downregulation of PASMC Kv1.5 in MCT-PAH

We found a significant decrease in K⁺ current in freshly isolated PASMC from the MCT-PAH rats, compared with the controls (Figure 6A,B), which was improved by the prevention and reversal DCA treatment protocols (Figure 6A, 6C). Most of the suppressed K⁺ current in the MCT-PAH PASMC is 4-aminopyridine-sensitive (ie, Kv current), with a much smaller component being iberiotoxin-sensitive (ie, large conductance calcium-activated, BK_{Ca} current). The decrease in the Kv was both prevented and reversed by DCA (Figure 6D).

Although many K⁺ channels are active in the PAs, Kv channels, and specifically Kv1.5 and Kv2.1, are the ones

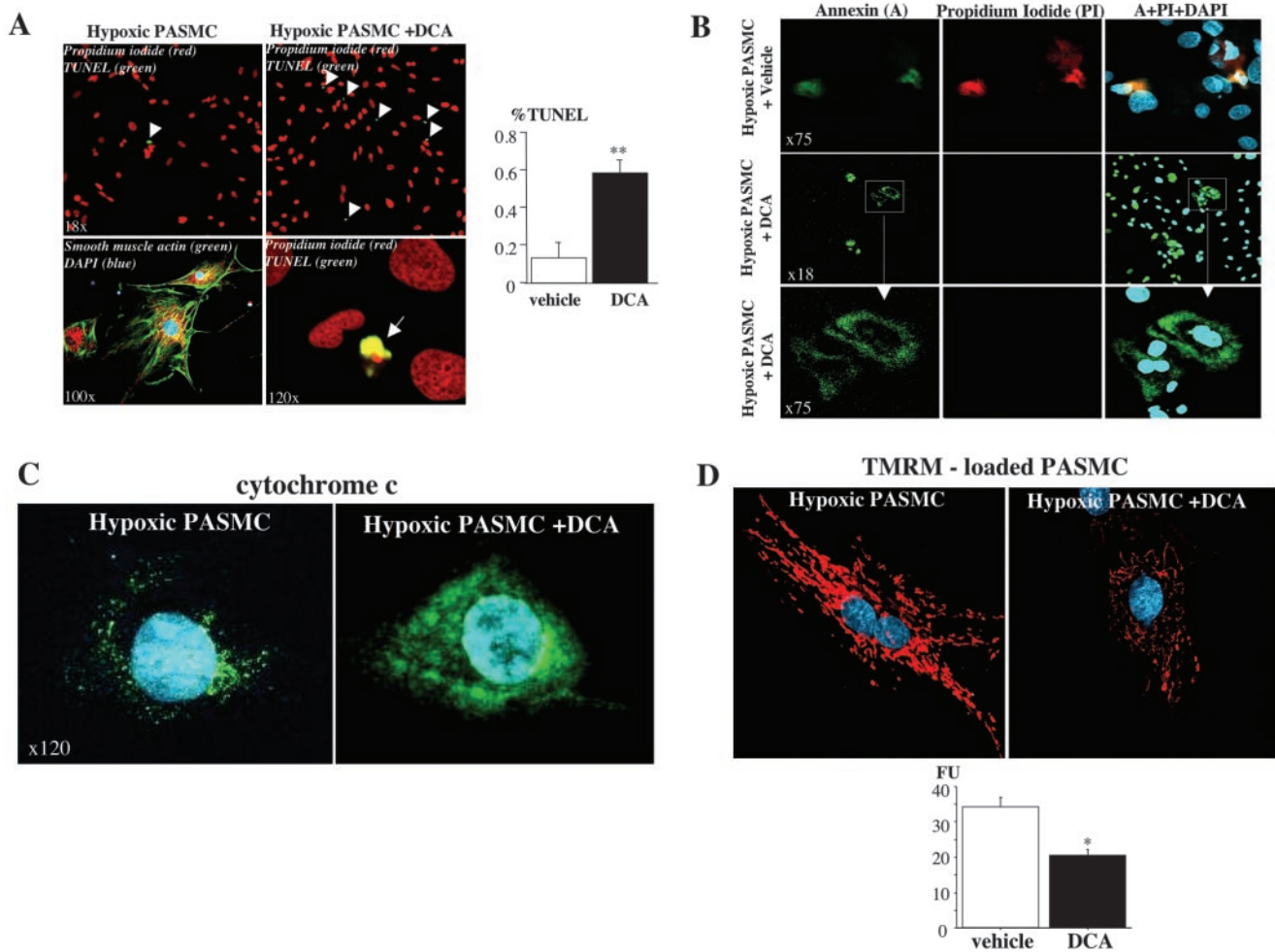


Figure 4. DCA induces mitochondria-dependent apoptosis in vitro. **A**, Hypoxic PASMCs in culture show low rates of apoptosis measured by TUNEL. Treatment with DCA 100 $\mu\text{mol/L}$ for 24 hours increases apoptosis. $*P < 0.05$ versus hypoxia + vehicle, $n \approx 20$ plates/group, 4 random fields/plate. Cells are identified as PASMC by their smooth muscle actin positivity (triple-stained with DAPI-blue, mitotracker-red and SMA-green). TUNEL-positive PASMC nuclei were shrunken and pyknotic, consistent with late stages of apoptosis. **B**, DCA (100 $\mu\text{mol/L}$ for 6 hours) induces expression of annexin on the plasma membrane of hypoxic PASMCs. Two necrotic cells are seen in the image of vehicle-treated cells (positive for both annexin and propidium iodide [PI]). The cells in the DCA-treatment panels are apoptotic because they are annexin-positive and PI-negative. **C**, Hypoxic vehicle-treated PASMCs do not show release of cytochrome c from mitochondria into the cytoplasm (discrete, punctate staining pattern). DCA treatment (100 $\mu\text{mol/L}$ for 6 hours) causes release of cytochrome c (diffuse staining of the cytoplasm). **D**, Vehicle-treated hypoxic PASMC mitochondria are hyperpolarized, whereas PASMC mitochondria from DCA-treated rats are depolarized, as shown by the reduced TMRM fluorescence ($n = 20/\text{group}$).

controlling PASMC membrane potential²¹ and are the ones primarily expressed in small resistance PAs.^{18,22} To molecularly identify the Kv current modulated by DCA treatment, we measured expression of protein and mRNA for Kv1.5 and Kv2.1. The protein levels of Kv1.5 and Kv2.1 channels in MCT-PAH PAs are decreased, compared with the controls (Figure 6E, 6F). DCA treatment almost completely reverses the downregulation of Kv1.5 but not Kv2.1 (Figure 6E, 6F), in agreement with our electrophysiology data.

Both Kv1.5 and Kv2.1 mRNA levels were significantly decreased in laser-capture microdissected resistance (25 to 50 μm) PAs of the MCT-PAH compared with the normal control rats (Figure 6G). DCA treatment partially reversed the downregulation of Kv1.5 ($P < 0.05$) but not Kv2.1 (although there was a trend, $P = 0.06$).

Changes in K⁺ Channels and Apoptosis Parallel the Hemodynamic Effects of MCT and DCA Treatment

To characterize the timing of the development of MCT-PAH and the temporal relation between the observed hemodynamic and molecular effects of MCT and DCA treatment, we performed additional time-course experiments. We used telemetry catheters and ECHO to prospectively measure hemodynamics, so that rats would serve as their own controls. At day 14, the time that our early reversal protocol begun, PAH is mild but clearly established, as shown by direct PA measurement with telemetry (Figure 7A) and PAAT (Figure 7B). That the PA pressure increase is significant is also shown by the fact that RVH is present (Figure 7B). We performed quantitative reverse-transcription polymerase chain reaction in whole lungs from these animals and we

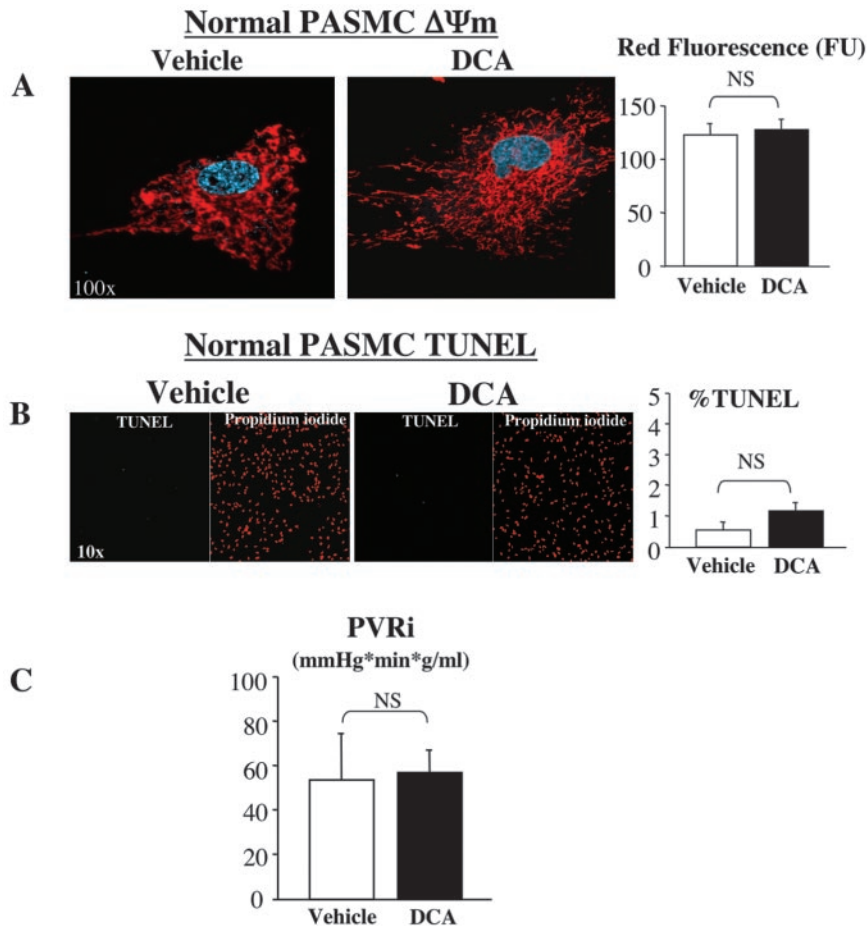


Figure 5. DCA has no effects on normal PASMCM. DCA does not affect $\Delta\Psi_m$ (n=20) (A) and apoptosis in normal PASMCM (n=10plates/group, 4 random fields/group) (B). C, DCA does not alter PVRi in normal rats (n=5/group).

show that Kv1.5 and Kv2.1 downregulation precedes the development of PAH (Figure 7C). Kv2.1 is maximally suppressed as early as day 7 after MCT and Kv1.5 is maximally suppressed at day 14; expression of both channels decreases minimally beyond that point, whereas the maximal effects in hemodynamics occur between day 14 and day 21. Both MCT and DCA had minimal and no significant effects on Kir2.1. In agreement with our protein expression and laser capture microdissection–quantitative reverse-transcription polymerase chain reaction data (Figure 6), these whole-lung data show that DCA treatment upregulates Kv1.5 but not Kv2.1.

We found a trend for a decrease in apoptosis during the first 2 weeks after MCT (which did not reach statistical significance). Apoptosis shows a tight temporal relation with both the hemodynamic and Kv channel effects of DCA because there is a 12-fold ($\approx 600\%$ increase, similar to our data in Figure 2C) in apoptosis from day 14 to day 21 (Figure 7C). The TUNEL-positive cells were all in the media and adventitia, as in Figure 2.

Discussion

We show that DCA can both prevent and reverse established pulmonary vascular remodeling in a common model of PAH and thus improve hemodynamics, RVH, and survival. We show that DCA increases the apoptosis/proliferation ratio in the media of remodeled PAs, without affecting healthy tissues

or systemic vessels. DCA depolarizes PASMCM mitochondria, thus initiating mitochondria-dependent apoptosis. DCA also reverses the downregulation of Kv current that occurs in MCT-PAH, and by this mechanism it further enhances apoptosis and suppresses proliferation (Figure 8). Our work shows for the first time to our knowledge that mitochondria-dependent apoptosis in the vascular wall is important for vascular remodeling and that the mitochondria and K^+ channel axis can be targeted therapeutically in PAH. Because DCA is selective for the pulmonary circulation, has a very good safety profile in PAH rats (Table I) and humans,¹³ and is orally available, it is a very attractive potential therapy for human PAH, a disease in which effective, simple to deliver, and nontoxic therapies are urgently needed.

The degree of DCA-induced apoptosis in the PA media is in agreement with a recent study showing that the regression of PA vascular remodeling caused by elastase inhibitors is associated with induction of apoptosis (measured by TUNEL) and a decrease in cell proliferation (measured by PCNA expression) in the media of the PAs.²⁰ Similarly, Nishimura et al recently showed that simvastatin caused regression of vascular remodeling by inducing vascular PASMCM apoptosis.²³ These two studies and our results confirm the hypothesis that PAH might be an apoptosis resistance state and that pharmacological enhancement of apoptosis would be therapeutically beneficial. In contrast, Zhao et al showed that apoptosis is increased in MCT-PAH and that suppressing

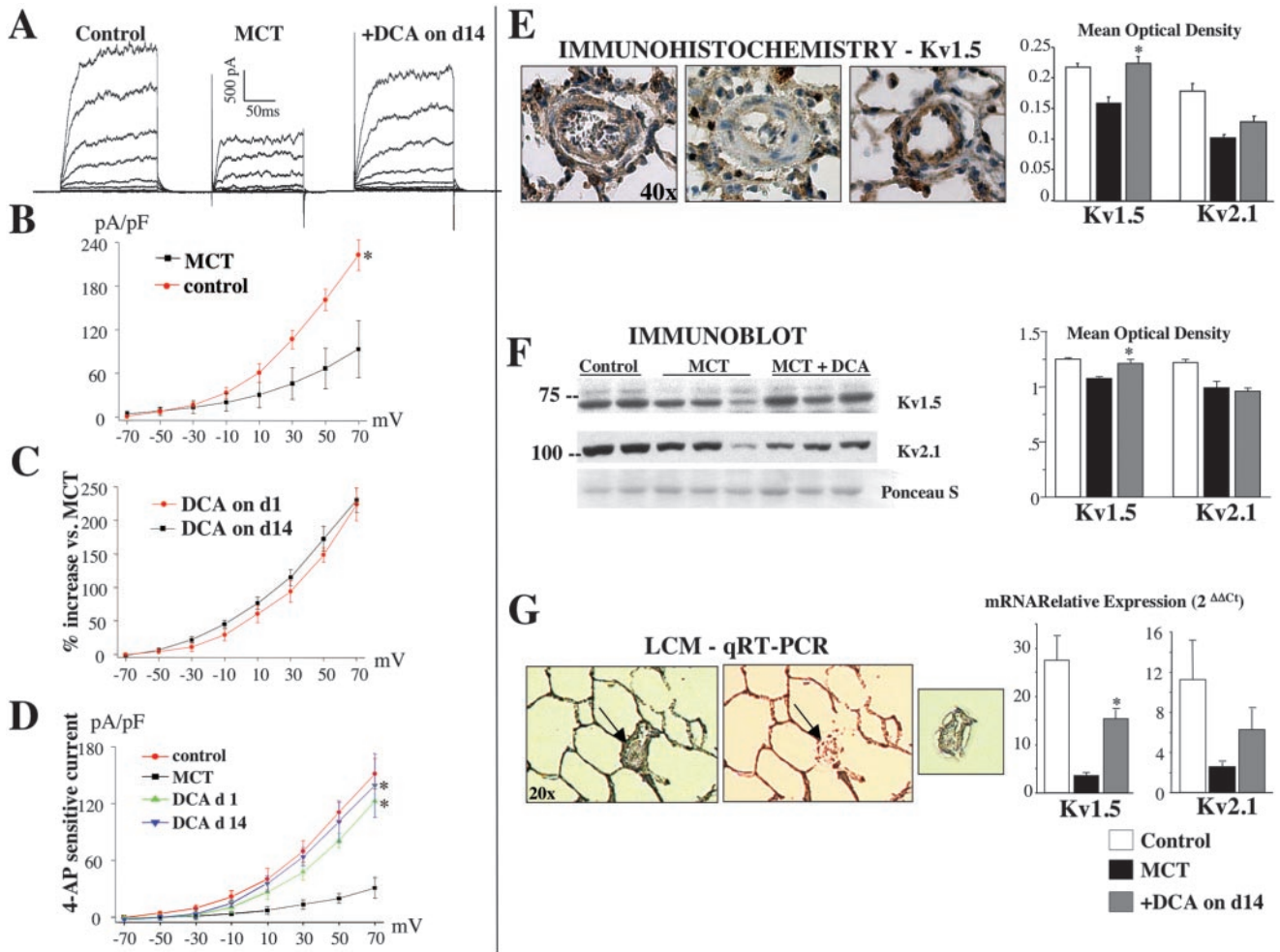


Figure 6. DCA reverses the Kv1.5 downregulation. A to D, PASMC from MCT rats ($n=10$) have markedly decreased K^+ current density, which is both prevented ($n=9$) and reversed ($n=5$) by DCA ($*P<0.05$ versus control). Representative traces and current density mean data are shown. The percent increase in the current is almost identical whether DCA is given on day 1 or on day 14. DCA normalizes the 4-AP-sensitive current, which is significantly suppressed in untreated MCT rats. E and F, Immunohistochemistry (left: control; middle: MCT-PAH; right: DCA-treated; Kv1.5 in brown) and immunoblots show Kv1.5 and Kv2.1 protein expression is decreased in the PAs of MCT rats, but only Kv1.5 is augmented by DCA ($*P<0.05$ versus MCT). G, Quantitative reverse-transcription polymerase chain reaction of laser-captured resistance PAs demonstrates that Kv1.5 and Kv2.1 mRNA is decreased in MCT, but only Kv1.5 is partially restored by DCA ($*P<0.05$ versus MCT, $n\approx 15$, 3 rats/group).

apoptosis with angiopoietin gene transfer prevented PAH.¹⁹ These data are not necessarily conflicting, because Zhao et al studied endothelial cell apoptosis and intervened early, when apoptosis in the endothelial cells in capillaries and very small PAs might indeed be increased, perhaps as a direct result of MCT, a known endothelial toxin. We focus on PASMC apoptosis and in our reversal protocol, we intervene late in already remodeled PAs. Intervening in established PAH is more relevant clinically, because patients typically present late. We did not find any evidence of increased apoptosis in the endothelial cells (at least on day 7 after MCT; Figure 7C), although this observation is limited by the fact that we did not use an endothelial marker to look for very small arteries and capillaries. More studies are needed to clarify the intriguing hypothesis that apoptotic mechanisms might show diversity within the vascular wall segments and might also vary with the age of the remodeling process.

Our findings that DCA depolarizes PASMC mitochondria early and causes release of mitochondrial cytochrome c

(Figure 4) provide a potential mechanism for the apoptosis that we observed in vivo. The initiation of mitochondria-dependent apoptosis can be further potentiated downstream by the decrease of intracellular K^+ that follows the DCA-induced activation of Kv channels⁷ and the upregulation of Kv1.5 expression. Kv1.5 might be a key player in PASMC and may regulate apoptosis, much like Kv2.1 modulates apoptosis in neurons.²⁴ Both the increase in cytochrome c and the decrease in intracellular K^+ explain the activation of caspase 3 that we observed in the PA (Figure 3A).

In human PAH, receptor-mediated apoptosis is suppressed, at least in part, because the BMPR-2 axis is suppressed. Cell culture studies indicate that BMP-2 normally causes K^+ channel-dependent apoptosis of SMCs, which may not occur in cells with the BMPR-2 mutation.⁴ In other words, K^+ channels modulate apoptosis distal to both receptor-mediated and mitochondria-mediated apoptosis. DCA can directly increase apoptosis and activate K^+ channels. Although both the increase in cytochrome c and H_2O_2 explain the activation

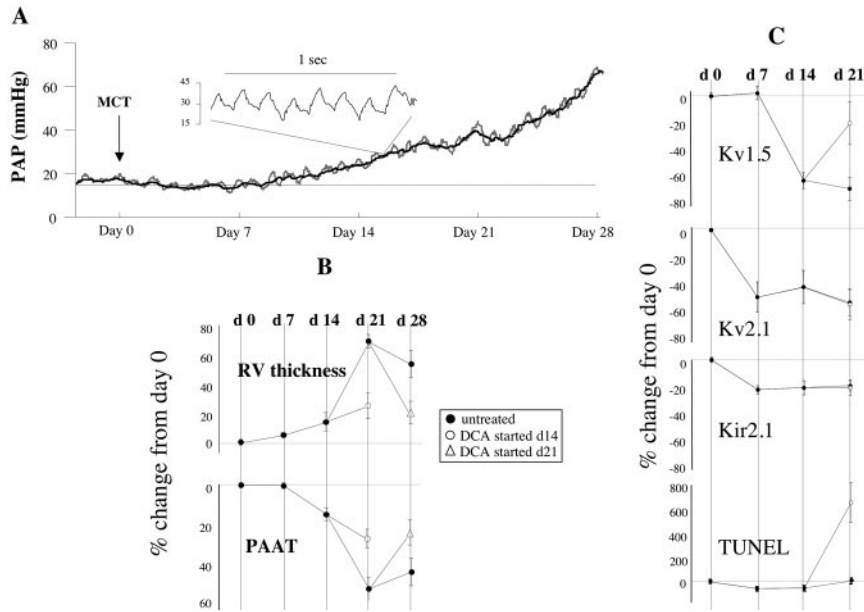


Figure 7. The K⁺ channel modulation and apoptosis closely parallel hemodynamics in MCT-PAH. A, Continuous recording of mean PA pressure in a freely moving untreated MCT-PAH rat with implanted telemetry catheter (representative of three). ECHO data (B), whole lung K⁺ channel expression (quantitative reverse-transcription polymerase chain reaction), and apoptosis (percent TUNEL-positive cells) (C) in the time course experiments.

of Kv channels, the mechanism by which DCA increases Kv1.5 mRNA expression, and whether this is related to its effects on mitochondria, remains unknown. However, this potential synergy of mechanisms (ie, mitochondria and Kv channels) might explain why DCA is so effective in PAH, normalizing PVRI and RVH (Figure 1). For example, we recently showed that simply replenishing Kv1.5 with gene therapy improved rat CH-PAH, but the effects on hemodynamics and RVH were only modest.¹⁵

How would DCA cause the opening of the mitochondria transition pore (MTP) and mitochondrial depolarization? MTP is redox-sensitive and an increase in electron transport chain production of AOS can cause the opening of the MTP and mitochondrial depolarization.¹⁰ DCA enhances mitochondrial oxidative phosphorylation by increasing the levels of intramitochondrial pyruvate and acetyl-CoA levels, which follows pyruvate dehydrogenase kinase inhibition.¹³ The increase in the acetyl-CoA that enters the Krebs cycle causes

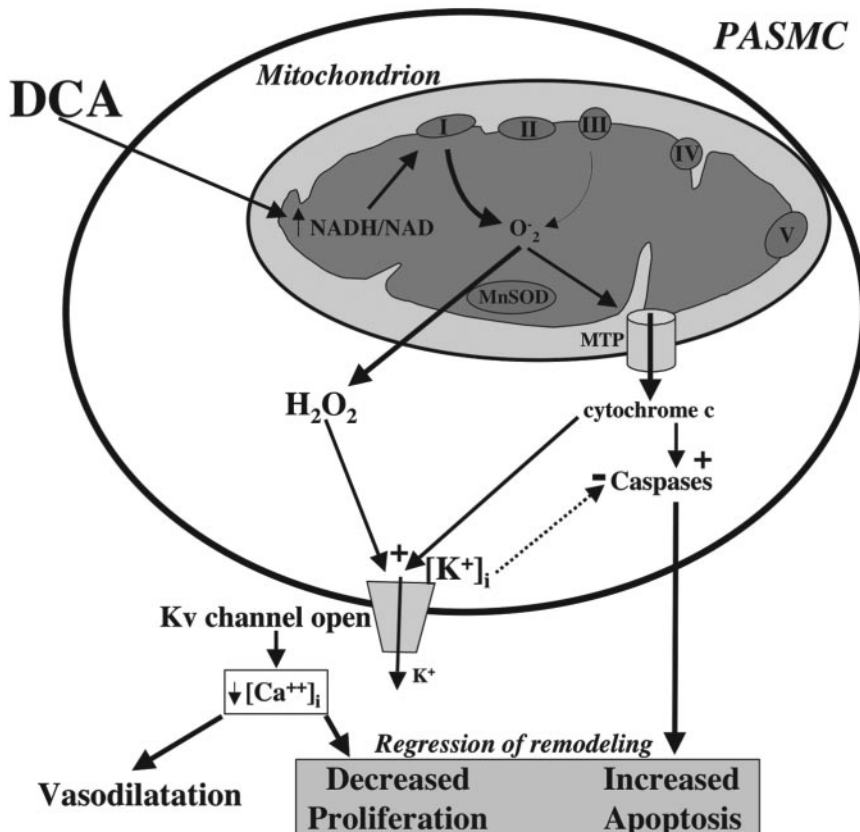


Figure 8. A proposed mechanism for the effects of DCA on pulmonary vascular remodeling.

an increase in the NADH/NAD ratio in the mitochondrial matrix, which in turn increases the AOS produced in the complex I of the ETC.²⁵ PASMCMitochondria have very high levels of MnSOD, compared with systemic SMC mitochondria, and thus superoxide is preferentially dismutated to H₂O₂,¹¹ a well-known stimulant of mitochondrial-dependent apoptosis and opener of the MTP.^{26–28} DCA acutely increases the production of H₂O₂ in PAs (Figure 3C and Figure III), and this increase is occurring in complex I of the ETC, because it is inhibited by rotenone (Figure 3C).

Although the doses that we used in vitro and in vivo are in agreement with the doses required to inhibit PDH kinase (and thus activate PDH), we have previously shown that at 10 μmol/L (a dose thought to be too small to inhibit PDH kinase) DCA activates expressed Kv channels in vitro and that this activation is tyrosine kinase-dependent.⁷ It is possible that DCA could have 2 mechanisms, involving tyrosine kinase at very low doses and PDH kinase at higher doses, although we cannot rule out PDH kinase isozymes in the resistance PASMCMitochondria with increased sensitivity to DCA.

Our proposed mechanism for the effects of DCA on mitochondria-derived H₂O₂, K⁺ channel function and apoptosis (Figure 8) resembles a recently proposed mechanism for HERG (a K⁺ channel important in the regulation of myocardial repolarization) on regulating apoptosis of tumor cells.²⁹ H₂O₂-induced apoptosis in tumor cell lines is suppressed by pharmacological or molecular inhibition of HERG.²⁹ A similar mechanism might occur in the “K⁺ channel-deficient” PAH, the primary form of which has been proposed to be a form of vascular neoplasia.³⁰ We recently showed that the PASMCMitochondria have relatively depolarized mitochondria, more MnSOD, and tonically produce more H₂O₂ compared with systemic vascular SMC mitochondria.¹¹ In PAH, the “protective” effects of H₂O₂ in both tone and apoptosis might be inhibited because of the deficiency of Kv channels, contributing to PASMCMitochondria contraction and proliferation.

DCA has no effects on normal PASMCMitochondria (Figure 5), in agreement with our previous report that DCA increases Kv current in hypoxic, but not normoxic, CHO cells expressing Kv2.1.⁷ This is also in agreement with the fact that DCA activates K⁺ current in myocardial cells from an infarcted area but not from healthy myocardium.³¹ This is important clinically because DCA might “target” mitochondria only in abnormal or proliferating PASMCMitochondria, minimizing possible toxicity to healthy cells and tissues.

Another clinically attractive property of DCA is that its effects are specific to the pulmonary circulation, because it does not alter systemic hemodynamics (Figure 1, Table I).⁷ Often the treatment of PAH patients is limited by the systemic hemodynamic effects of therapies, causing hypotension. The mechanism for the selectivity of DCA to the pulmonary circulation is unknown, but the differences of the PASMCMitochondria compared with systemic SMC mitochondria¹¹ raise the possibility that PASMCMitochondria are more sensitive to the effects of pyruvate dehydrogenase kinase inhibition.

The present study and our recent studies⁷ suggest that DCA is an attractive treatment for human PAH and provide the rationale for initiation of a clinical trial in humans with PAH.

Our study also suggests that the interplay of mitochondria, membrane Kv channels, and apoptosis might provide novel pharmacological targets for the treatment of vascular disease.

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