Microtubule Depolymerization Normalizes In Vivo Myocardial Contractile Function in Dogs With Pressure-Overload Left Ventricular Hypertrophy

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Background—Because initially compensatory myocardial hypertrophy in response to pressure overloading may eventually decompensate to myocardial failure, mechanisms responsible for this transition have long been sought. One such mechanism established in vitro is densification of the cellular microtubule network, which imposes a viscous load that inhibits cardiocyte contraction.

Methods and Results—In the present study, we extended this in vitro finding to the in vivo level and tested the hypothesis that this cytoskeletal abnormality is important in the in vivo contractile dysfunction that occurs in experimental aortic stenosis in the adult dog. In 8 dogs in which gradual stenosis of the ascending aorta had caused severe left ventricular (LV) pressure overloading (gradient, 152±16 mm Hg) with contractile dysfunction, LV function was measured at baseline and 1 hour after the intravenous administration of colchicine. Cardiocytes obtained by biopsy before and after in vivo colchicine administration were examined in tandem. Microtubule depolymerization restored LV contractile function both in vivo and in vitro.

Conclusions—These and additional corroborative data show that increased cardiocyte microtubule network density is an important mechanism for the ventricular contractile dysfunction that develops in large mammals with adult-onset pressure-overload–induced cardiac hypertrophy. (Circulation. 2000;102:1045-1052.)

Key Words: heart failure ▪ hypertrophy ▪ stenosis ▪ cells ▪ microtubules

If the extent of cardiac hypertrophy in response to pressure overloading is insufficient to normalize wall stress, afterload mismatch reduces ejection performance,1 and increased stress itself becomes an intrinsically important determinant of the transition to depressed myocardial contractility and heart failure.2 Causes proposed for this transition to pathological hypertrophy have included reduced coronary blood flow resulting in subendocardial ischemia,3–5 altered calcium metabolism,6,7 and replacement fibrosis of the myocardium.8 More recently, we have implicated increased density of the cardiocyte microtubule network as a cause for contractile impairment in pressure-overload hypertrophy,9 in which microtubules act as a viscous element inhibiting sarcomere shortening.10 We have validated this hypothesis in cardiocytes from pressure-overloaded right ventricles (RVs) exhibiting either compensated hypertrophy11 or decompensated failure12 and ascribed the increased microtubules both to increased tubulin synthesis13 and to increased stability14 of the microtubules once formed. Most importantly, microtubule depolymerization after either colchicine or cold exposure returned cardiocyte contractile function to normal,9 and we have recently extended this finding to the tissue level, where microtubule depolymerization restored normal contractile function to initially quite abnormal papillary muscles from pressure-overloaded feline RVs.15 This cytoskeletal alteration has tightly restricted specificity to pressure-overload hypertrophy imposed in the adult mammal that causes persistently increased ventricular wall stress, because, as we have reviewed in some detail elsewhere,15 neither we nor others have found it in other abnormal cardiac muscle states.

Because the most important consequence of hypertrophic decompensation in adult humans is left ventricular (LV) rather than RV dysfunction, the present study was undertaken to examine this mechanism, defined originally in vitro at the cellular and tissue levels in the RV and in the pressure-overloaded LV at the clinically relevant in vivo level.
Methods

Study Design
Progressive LV pressure-overload hypertrophy was induced in 12 mongrel dogs by an adjustable band placed around the proximal ascending aorta. After 8 weeks, 8 dogs developed LV dysfunction and constituted the study group. At that time, the pressure gradient was relieved, and LV function was remeasured. One hour later, colchicine (1 mg/kg IV in 100 mL normal saline) was administered, and LV function was measured again. This dose of colchicine is the minimum needed for full in vivo microtubule depolymerization in 1 hour, as validated by myocardial immunoblots. Each dog served as its own control, but 7 normal dogs in which LV function was measured before and after colchicine administration served as additional controls.

Assessment of LV Contractility
The hypothesis being tested was that depressed contractility in pressure-overloaded LVs would improve via experimentally induced depolymerization of cardiocyte microtubules. Therefore, it was incumbent on us to have an in vivo instrument for determining contractility. The ideal contractility index would be independent of load and heart size, but because no universally accepted single index such as this exists, we applied several approaches to assessing cardiac performance in vivo. Figure 1A demonstrates the normal relationship between mean normalized systolic ejection rate (MNSER) and mean systolic LV wall stress derived previously from 40 normal β-blocked dogs. In this paradigm, relatively preload-insensitive MNSER is plotted against and thus normalized for afterload. A plot of a ventricle that falls below and to the left of the normal relationship indicates reduced extent or velocity of contraction for any given afterload, denoting contractile impairment. However, because MNSER records endocardial events, which might overestimate contractility of hypertrophied ventricles, we also analyzed modified midwall mean velocity of circumferential fiber shortening ($c_{mm}V_c$) plotted against mean systolic wall stress (Figure 1B). The dogs were lightly anesthetized with a droperidol-fentanyl combination given by inhalation, an anesthetic mix shown previously not to impair LV function. To prevent the confounding influences of changing adrenergic tone on inotropic state and heart rate during our estimations of contractility, all studies were done during β-blockade induced by a constant infusion of esmolol (0.3 mg kg$^{-1}$ min$^{-1}$), a dosage validated before in dogs by isoproterenol challenge.

Rationale for Gradient Ablation Before Colchicine Administration
It was anticipated that if increased microtubule network density indeed had a role in contractile dysfunction in this model, microtubule depolymerization would increase cardiac output, which in the presence of severe LV outflow obstruction would increase the pressure gradient, in turn inhibiting LV contraction and possibly inducing ischemia. This would obscure any ameliorative effects of microtubule depolymerization. Furthermore, removal of the effects of microtubule-based viscous loading would be minimally apparent during isometric contraction, which is akin to the high-afterload state of severe aortic constriction. Although balloon deflation with removal of the gradient would decrease LV afterload and enhance LV ejection performance, if ejection performance remained depressed even after afterload normalization, it would further support the hypothesis that depressed contractility independent of excess afterload was present before colchicine administration. Finally, afterload reduction alone would cause parallel shifts in the MNSER-afterload and the $c_{mm}V_c$-afterload relationships, with improved ejection performance as afterload decreased (Figure 1, lines 1→2). In this case, however, the relationship would still be outside the normal range. Any improvement in contractile function after the administration of colchicine would be expected to cause an increase in LV ejection performance disproportionately greater than any change in afterload.

Confirmatory In Vivo Versus In Vitro Studies
The tacit assumption of the present study was that if colchicine, which our own data show to be a noninotropic agent in normal adult cardiocytes and myocardium, improved contractile function of hypertrophied myocardium, the mechanism for restored contractility would be microtubule depolymerization. To confirm that this was the case, isolated cardiocyte function and cardiocyte microtubule network density were examined in an LV biopsy taken before in vivo colchicine administration at the same time that in vivo LV function was examined. Cardiocyte function and microtubule network density were examined again after in vivo colchicine administration just after the terminal study of LV function. Thus, in these studies, in vivo contractile function before and after colchicine was correlated with in vitro cellular function and microtubule network density.

Confirmatory Studies Using Hypothermia
The intent here was to examine the effects on LV contractile dysfunction of microtubule depolymerization induced by a means...
distinct from colchicine and thus with presumably quite different secondary effects. Because microtubules fully depolymerize on exposure to a temperature of $<10^\circ$C for 1 hour and repolymerize on rewarming, we reversibly cooled the hearts of intact dogs with either severe or a more moderate degree of aortic stenosis.

For this purpose, at 8 weeks after aortic banding, dogs were anesthetized, instrumented, and $\beta$-blocked as above before left ventriculograms, first with the aortic gradient present and then after it was removed. An epicardial LV biopsy was then performed. The dogs were next submitted to closed-chest cardiopulmonary bypass with cold-blood coronary artery perfusion. An endovascular occlusion balloon at the aortic sinotubular junction that allowed coronary artery perfusion was inflated, and blood at $<4^\circ$C was delivered antegrade at $\approx 250$ mL/min through the balloon catheter. Myocardial temperature was kept at $<10^\circ$C. After 60 minutes of cold cardiac arrest, a second LV biopsy was performed, the balloon was deflated, and the heart was rewarmed; after both myocardial and core body temperatures had returned to normal, the animal was weaned from cardiopulmonary bypass. Three hours later, a third left ventriculogram and LV biopsy were performed.

Calculations
LV mass at baseline and at terminal study was calculated angiographically by the method of Rackley et al. Mass at terminal study was also obtained by direct weight. Systolic wall stress was calculated on a frame-by-frame basis by Mirsky’s formula. Stresses from the total number of systolic frames were then averaged to yield mean systolic wall stress, which was used to derive the relationships between MNSER and wall stress and between $c_{mm} V_{cf}$ and wall stress. The formulas that we use for $c_{mm} V_{cf}$ and its derivation are given elsewhere.

Results
LV mass corrected for body weight increased in the aortic stenosis group from $4.36 \pm 0.22$ to $7.28 \pm 0.36$ g/kg ($P<0.001$) at 8 weeks. Peak pressure gradient with no $\beta$-blockade increased from 0 to $152 \pm 16$ mm Hg. Ejection fraction fell from $0.58 \pm 0.06$ to $0.39 \pm 0.03$ ($P<0.01$). After balloon deflation, the pressure gradient during $\beta$-blockade fell from $78 \pm 10$ to $20 \pm 2$ mm Hg. Colchicine caused a slight but insignificant rise in the pressure gradient. As expected, mean wall stress fell from $205 \pm 20$ to $120 \pm 30$ kdyne/cm² after balloon deflation. After colchicine administration, because of a modest increase in LV pressure, there was a slight but insignificant increase in wall stress.

Changes in LV ejection performance are shown in Figure 2A, and the values for selected hemodynamic variables during these measurements are given in the Table. Ejection fraction increased insignificantly from $40 \pm 3\%$ to $47 \pm 4\%$ but was still depressed after balloon deflation. Ejection fraction then rose significantly to $61 \pm 7\%$ after colchicine administration, such that ejection fraction was now significantly greater than either at the beginning of the terminal study or after release of the balloon. In fact, ejection fraction now was no different from that at baseline before banding. Although increased contractility, decreased afterload, or increased preload all could have increased ejection fraction, the primary mechanism by which ejection fraction rose is shown in Figure 2B. End-systolic volume fell as expected with balloon deflation and afterload reduction, but this fall was insignificant. After colchicine, however, there was a greater and significant reduction in end-systolic volume with no change in end-diastolic volume, or preload. Together with the slight increase in wall stress shown in the Table and Figure 3A after administration of colchicine, this significant fall in end-systolic volume indicates an increase in contractility. Further studies of LV contractility are shown in Figure 3. After balloon deflation in the aortic stenosis dogs (Figure 3A), there was an increase in $c_{mm} V_{cf}$ in parallel with the decrease in wall stress. The relationship after balloon deflation remained far down and to the left of the normal relationship, as it was at baseline. However, after administration of colchicine, $c_{mm} V_{cf}$ increased significantly despite a small increase in afterload, such that it fell at the lower limit of the normal relationship. In contrast, Figure 3B shows that administration of colchicine to normal dogs depressed rather than augmented LV contractile function. Furthermore, 1 mg/kg of lumicolchicine, the biologically inactive stereoisomer of colchicine, had no effect on any aspect of LV function in either aortic stenosis or normal control dogs.

Correlative Substudies
Ventricular Versus Cellular Contractile Function and Microtubule Content
Data from the biopsied animal are shown in Figure 4. Before colchicine was administered in vivo, a myocardial biopsy

![Figure 2. LV ejection performance at final study 8 weeks after initial aortic banding. A, Ejection fraction in aortic stenosis dogs at baseline, after release of aortic band, and after administration of colchicine. B, End-systolic volume in aortic stenosis dogs at same times. Statistical comparisons were by 1-way ANOVA followed by Scheffe’s S procedure, where $n=8$. †$P<0.05$ for difference from Baseline value. *$P<0.05$ for difference from Band Released value.](http://circ.ahajournals.org/doi/abs/10.1161/CIRCULATIONAHA.117.031321?journalCode=circ)
showed increased microtubule network density (B) compared with that in a normal dog (A). Cardiocyte function was depressed (C) but returned to normal after in vitro colchicine (D). However, 1 hour after in vivo colchicine, microtubule network density was greatly decreased both in a normal (E) and in an aortic stenosis (F) dog, and cardiocyte function was normal in the aortic stenosis dog (G) and did not improve further with in vitro colchicine (H), because in vivo colchicine had already normalized function.

**Colchicine-Independent Microtubule Depolymerization**

The effects of myocardial hypothermia are shown in Figure 5. Where our value for normal dogs is 169.1 ± 15.5 kdyne/cm², the dog with severe aortic stenosis and high baseline wall stress had an increased proportion of tubulin in the microtubule pool, as seen before in this model, whereas the dog with moderate aortic stenosis and a lesser baseline wall stress increase had the expected normal 2:1 ratio of free:polymerized tubulin. Both dogs, cooling the myocardium to <10°C for 1 hour caused nearly complete microtubule depolymerization. With myocardial rewarming, the ratio of free:polymerized tubulin returned to 2:1 for both dogs, just as we have found for cardiocytes isolated from pressure-overloaded myocardium. LV contractility in the dog with severe aortic stenosis was borderline abnormal initially and distinctly abnormal after aortic stenosis was relieved, despite the reduction in wall stress. After microtubule depolymerization and rewarming, contractility clearly improved, despite the depression of contractility within this time frame associated with hypothermic cardiac arrest, as shown in Figure 5B for the dog with moderate aortic stenosis whose initially normal LV contractile function became quite abnormal after these interventions. Note that in contrast to Figure 3, for both dogs, LV wall stress fell further after relief of aortic stenosis as a result of the peripheral vasodilation attendant on cardiopulmonary bypass.

**Time-Dependent Effects of Aortic Gradient Ablation on LV Function**

Because removal of the aortic pressure gradient would be expected to have the eventual ameliorative effect on LV function that is observed after corrective surgery for aortic stenosis, it was necessary to determine whether such an effect could be detected within the time frame of our experimental interventions. Therefore, in a dog with severe aortic stenosis and high LV wall stress at 8 weeks after initial aortic banding, whose abnormal baseline LV function was coincident with point 1 in Figure 3A, the aortic pressure gradient was removed, such that the relationship between cff and mean systolic wall stress moved to a value coincident with point 2 in Figure 3A. No further intervention was imposed. At 4 hours after aortic gradient ablation, a time considerably longer than that at which the effects of colchicine administration were fully realized, the relationship between cff and mean systolic wall stress was by 1-way ANOVA followed by Newman-Keuls f test. *P<0.05 for difference from Baseline.
and mean systolic wall stress was unaltered from point 2 in Figure 3A.

**Discussion**

Finding the causes of LV dysfunction during pressure overloading, which stems from both afterload excess and impaired contractility, is of apparent importance. We have recently identified an increase in the microtubule component of the extramyofilament cardiocyte cytoskeleton as one of these causes. Data from these studies that support this statement are as follows. First, in accordance with Koch’s first and second postulates, the microtubule increase is absent from both afterload excess and impaired contractility. Second, in accordance with Koch’s third postulate, agents that increase microtubules independently of hemodynamic input were shown in these studies to reproduce the contractile and cytoskeletal abnormalities seen in severe pressure-overload cardiac hypertrophy.

Thus, microtubule depolymerization ameliorates contractile dysfunction in previously impaired cardiocytes and papillary muscles from pressure-overload-hypertrophied feline RVs, in which we have found that increased density of the microtubule network is apparently based on upregulation of a fibrous microtubule-stabilizing protein, MAP 4, and a switch in the expression pattern of the β-tubulin multigene family. These findings were confirmed in cardiocytes from the pressure-overload-hypertrophied canine LV in our hands and from the guinea pig LV in the hands of others. But whether data derived from cells and tissue in vitro are applicable in vivo to the LV of a large mammal having a
Functionally, pressure-overload hypertrophy is characterized by an increase in ventricular wall stress that precedes any measure of contractility reduction. A, Dog with severe aortic stenosis (LV mass, 7.9 g/kg); B, dog with moderate aortic stenosis (LV mass, 5.9 g/kg), both at 8 weeks after initial aortic banding. A, Data first obtained immediately after final ventriculogram. Myocardial hypertrophy imposed on the adult heart that causes a persistent increase in ventricular wall stress. Just as clearly, therefore, the numerous other abnormalities of hypertrophied myocardium must be responsible for the myocardial dysfunction seen in the many other forms of pathological cardiac hypertrophy.

The central finding both in this and in our previous functional studies of the extramyofilament cardiocyte cytoskeleton is that microtubule depolymerization ameliorates contractile dysfunction in severe pressure-overload cardiac hypertrophy. However, the multiple roles of microtubules in interphase cells must raise some concern about the effects of microtubule depolymerization on properties other than apparent viscosity, defined by our data as cytoskeletal frictional dissipation rather than cytoplasmic viscosity, even within the short time domain of this experimental intervention. That is, might contractile function be altered by microtubule depolymerization and/or by the agents used to cause it in a manner independent of decreased structural damping attributable to the microtubule component of the cytoskeleton when excessive microtubules are removed from pressure-overload-hypertrophied cells or tissue? One potential concern, given that microtubule-based motors are important for a number of intracellular transport processes, including that of activated β-adrenergic receptors, is any inotropic consequence of altered β-adrenergic receptor activity. However, this concern is inconsequential for our previous in vitro studies and was obviated in the present in vivo study by the use of β-receptor blockade. But a more substantive concern, in view of the fact that we see a positive inotropic effect of microtubule depolymerization in pressure-overload cardiac hypertrophy, is calcium homeostasis. Here, our work provides direct evidence in both normal and hypertrophied hearts, and at the levels of the cardiocyte cytoskeleton itself and of the whole cell, that extramyofilament microtubule-based viscous damping is not altered by changes in [Ca2+].

This does not, however, speak to possible myofilament-based direct inotropic effects of our experimental interventions on calcium homeostasis. In this regard, 1 study has shown that microtubule depolymerization by colchicine causes an increase in the time that the L-type calcium channel in embryonic chick cardiocytes spends in the closed state, and taxol does the opposite, such that colchicine should have if anything a negative inotropic effect in normal cardiocytes, and there are other data showing that calcium release from intracellular stores is decreased by colchicine. It has also been shown in adult cardiocytes that neither colchicine nor taxol
has a direct effect on the L-type calcium channel in terms of voltage-dependent parameters. But quite recently, in contrast, it was reported that colchicine increases I_{Ca} transient in adult cardiocytes. However, the posited explanation for these effects, that αβ-tubulin heterodimers act as a functional analogue of G proteins to activate adenyl cyclase when their concentration is increased by colchicine, is difficult to accept, because other data in this same study show that taxol, which markedly reduces the cardiac αβ-tubulin heterodimer concentration, is without effect on these same cardiac variables. In our own previous in vitro study and present in vivo (Figure 4B) work, we find that with hypertrophy, there is, along with an increase in microtubules, a very significant and persistent increase in the concentration of free αβ-tubulin heterodimers, yet in contrast to what this mechanism would predict if it has functional significance, there is a marked decrement rather than increment in contractile function. Furthermore, we find that colchicine increases neither resting nor peak activated calcium, nor the rate of rise or fall of intracellular calcium in normal or hypertrophied cardiocytes and that colchicine increases neither cAMP, peak activated calcium, nor the rate of rise or fall of intracellular calcium in normal or hypertrophied myocardium. Most pertinent, however, to any consideration of potentially direct inotropic effects of microtubule depolymerization on contractile function is the fact that although we consistently find only a 5% to 10% increase in the extent and velocity of shortening of sarcomeres, cells, and tissue from normal hearts, as well of as the normal heart itself in vivo after microtubule depolymerization by any means, we find a much greater response to purposive inotropic interventions. Thus, although the relevant studies reach conflicting conclusions, colchicine may well have subtle effects on calcium homeostasis. But the functional significance of such findings in the context of the extensive changes in cardiac mechanics caused by microtubule depolymerization in severely pressure-overload–hypertrophied myocardium is open to quite substantial question.

Thus, this data set and the conclusions we draw from it are equivalently straightforward. Contractile dysfunction due to increased microtubule network density improves after microtubule depolymerization. To substantiate this conclusion, we must be certain that contractile dysfunction was actually present, that function improved, and that changes in the microtubules were causative of this improvement. It is clear that after 8 weeks of pressure overload, these canine LVs exhibited contractile dysfunction. Ejection performance either at the endocardium or at the midwall was reduced much more than was predicted from afterload excess alone. Indeed, when afterload was returned to normal by balloon deflation, ejection performance gauged by ejection fraction, by MNSER, or by midwall shortening rate was still quite depressed. However, microtubule depolymerization by colchicine returned ejection performance toward or to normal (Figures 2 and 3), and this finding was confirmed by the mechanistically independent means of cold-induced microtubule depolymerization (Figure 5). Furthermore, we believe that the data obtained in our parallel in vitro study (Figure 4) are extremely compelling. At a time when contractility was depressed in vivo, the contractility of cardiocytes taken from the same ventricle was also depressed. Increased microtubule network density was present in these cardiocytes, and its reduction by in vitro colchicine returned the function of the cardiocytes to normal. Hours later, after colchicine had been administered in vivo and ventricular function had returned to normal, cardiocyte function had also returned to normal, and increased microtubule network density was no longer present.

Therefore, for this clinically relevant form of substantial cardiac pressure overloading leading to myocardial dysfunction, the fact that removal of the dense microtubule network returned depressed contractile function to normal supports our hypothesis that this cytoskeletal abnormality plays an important role in this dysfunction in vivo.

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