Myocyte cytoskeletal disorganization and right heart failure in hypoxia-induced neonatal pulmonary hypertension

MATTHEW S. LEMLER,1* ROGER D. BIES,3* MARIA G. FRID,2 AMORNRATE SATAVACHA,3 LAWRENCE S. ZISMAN,3 TERESA BOHLMEYER,3 A. MARTIN GERDES,4 JOHN T. REEVES,2,3 AND KURT R. STENMARK2

1Division of Cardiology and 2Division of Critical Care and Developmental Lung Biology, Department of Pediatrics, and 3Division of Cardiology, Department of Medicine, University of Colorado Health Sciences Center, Denver, Colorado 80262; and 4Department of Anatomy and Structural Biology, University of South Dakota School of Medicine, Vermillion, South Dakota 57069

Received 12 October 1999; accepted in final form 21 February 2000

Lemler, Matthew S., Roger D. Bies, Maria G. Frid, Amornrate Satavacha, Lawrence S. Zisman, Teresa Bohlmeyer, A. Martin Gerdes, John T. Reeves, and Kurt R. Stenmark. Myocyte cytoskeletal disorganization and right heart failure in hypoxia-induced neonatal pulmonary hypertension. Am J Physiol Heart Circ Physiol 279: H1365–H1376, 2000.—Previous studies have demonstrated that environmentally or genetically induced changes in the intracellular proteins that compose the cytoskeleton can contribute to heart failure. Because neonatal right ventricular myocytes are immature and are in the process of significant cytoskeletal change, we hypothesized that they may be particularly susceptible to pressure stress. Newborn calves exposed to hypobaric hypoxia (barometric pressure = 430 mmHg) for 14 days developed severe pulmonary hypertension (pulmonary arterial pressure = 101 ± 6 vs. 27 ± 1 mmHg) and right heart failure compared with age-matched controls. Light microscopy showed partial loss of myocardial striations in the failing neonatal right but not left ventricles and in neither ventricle of adolescent cattle dying of altitude-induced right heart failure. In neonatal calves, immunohistochemical analysis of the cytoskeletal proteins (vinculin, metavinculin, desmin, vimentin, and cadherin) showed selectively, within the failing right ventricles, patchy areas characterized by loss and disorganization of costameres and intercalated discs. Within myocytes from the failing ventricles, vinculin and desmin were observed to redistribute diffusely within the cytosol, metavinculin appeared in disorganized clumps, and vimentin immunoreactivity was markedly decreased. Western blot analysis of the failing right ventricular myocardium showed, compared with control, vinculin and desmin to be little changed in total content but redistributed from insoluble (structural) to soluble (cytosolic) fractions; metavinculin total content was markedly decreased, tubulin content increased, particularly in the structural fraction, and cadherin total content and distribution were unchanged. We conclude that hypoxic pulmonary hypertensive-induced neonatal right ventricular failure is associated with disorganization of the cytoskeletal architecture.

cytoskeleton; costameres; intercalated disks; cor pulmonale; vinculin; metavinculin; desmin; cadherin; tubulin

Perhaps more than any other species, cattle, particularly when young, are susceptible to the development of severe pulmonary hypertension and subsequent right heart failure when exposed to hypoxia (10, 35, 43, 50). Little is known regarding the mechanisms that contribute to right heart failure, particularly when the stress inducing the failure is imposed in the immediate neonatal period. There are data to suggest the response to stress in the neonatal myocardium may be different from that in the adult myocardium, although most investigations have focused on the failing neonatal left ventricle. For example, compared with that of the adult, the left ventricle of the neonate has little reserve and thus fails to maintain its contractility in response to acute pressure overload (2, 4, 52). Furthermore, in the newborn, the cardiac myocytes are smaller and the myofibrils are less dense (2, 48), myocyte contraction is both slower and smaller in magnitude (2), myocardium has a less compliant state (32), genetic control and myocyte content of contractile proteins are changing (33, 46), autonomic innervation is immature (25), myocyte replication continues for some days after birth (26, 28, 33), and the intercalated disks (40) and microtubule composition and function are immature (59). These reports suggest that immaturity in ventricular structure and function could make calves susceptible to pressure overload imposed soon after birth. The neonatal right ventricle may be even more susceptible to chronic pressure overload than the left ventricle. Normally, not only is ventilation and normoxia rapidly unloading the right ventricle, but also concomitantly continued growth of the right ventricular wall begins to cease, whereas that of the left ventricle continues (44). Collectively, this evidence led us to hypothesize that right ventricular myocytes might not tolerate the severe and sustained pressure overload that occurs during and actually interrupts the normal postnatal right ventricular pressure fall in animals exposed to hypoxia shortly after birth.

*Authors contributed equally to the work presented.

Address for reprint requests and other correspondence: K. R. Stenmark, Developmental Lung Biology Research Laboratory, B-131, Univ. of Colorado Health Sciences Center, Denver, CO 80262 (E-mail: kurt.stenmark@uchsc.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Heart failure is characterized by a ventricular dilation and lengthening of individual cardiomyocytes (15). Although the molecular basis for this pathological response is unknown, several studies have implicated an important role for the intracellular structural elements that comprise the cytoskeleton (12). Several observational studies and genetic models in humans now suggest that cytoskeletal defects occur in cardiomyopathies and that, in some circumstances, such defects contribute to heart failure (5, 30, 45). In adult animal models of pressure overload, there is evidence that the cytoskeleton can respond to physiological stress and that the resultant changes can affect cell function (21, 56). Costameres and intercalated disks are cytoskeleton attachment sites that both support the myocyte framework and establish mechanical coupling within the ventricular myocardium. This linkage occurs via a complex of cytoskeletal proteins, including vinculin, metavinculin, and α-actinin (3, 30, 39), which are linked to cadherin at intercalated disks or the integrin receptors at costameres. Intermediate filament proteins, such as desmin, are also present and stabilize sarcomeres at the Z bands and intercalated disks at the desmosome (36, 37). Little is known of the changes that occur in these cytoskeletal attachment sites or in the distribution and abundance of the proteins that make up these sites during the process of cellular remodeling in acute pressure overload-induced right heart failure in the neonate.

We hypothesized that neonatal right heart failure secondary to acute and severe pulmonary hypertension would be associated with changes in cytoskeletal attachment sites in cardiac myocytes and thus would provide a cellular basis for ventricular remodeling. We further reasoned that the neonatal cardiac cytoskeleton might be particularly susceptible to stress-induced changes, especially when the stress was dramatic and imposed during the period of adaptation to extraterrestrial life, because the structural organization of the myocyte appears to continue to develop for some time after birth (9, 17, 40). Our approach was to describe in neonatal calves the in vivo response of the right ventricular myocyte to chronic hypoxia-induced pressure overload, a model that induces a far more acute and dramatic pressure overload than is observed in rodents exposed to the same chronic hypoxic stimulus. The present study involved documentation of the heart failure, a comparison by light microscopy of the failing right ventricle with the left ventricle in the same calves and with both ventricles of control calves, morphometric examination of isolated myocytes, immunohistochemical analysis of the cytoskeletal proteins known to be important in myocyte function (vinculin, metavinculin, desmin, vimentin, catheren, and tubulin), and Western blot analysis of total content of these proteins and (for some of them) their cellular distribution. Older cattle, which had died of right heart failure at high altitude (1), had routine histological sections of the myocardium that were compared by light microscopy with sections from the neonatal calves. The importance of these studies lies in our contention that insight into how the neonatal right ventricular myocyte responds to chronic severe pressure overload stress will facilitate an understanding of postnatal cardiac development and could also assist in the care of right heart failure in newborn children.

MATERIALS AND METHODS

Animal Model, Hemodynamics, and Echocardiography

Eighteen newborn male Holstein dairy calves were obtained from the Duo Dairy (Fort Collins, CO). Nine calves were chosen at random for the right heart failure group after they had nursed to obtain colostrum (24–48 h). In this group, pulmonary hypertension was induced through hypobaric hypoxia by placing them in a large (3 m × 9 m) hypobaric chamber located in the Department of Physiology, Colorado State University (Fort Collins, CO). They were maintained at altitude (barometric pressure = 430 mmHg, ~4,570 m above sea level) for 14 days, after which hemodynamic measurements were made while at altitude (this group will be referred to as high-altitude calves) (10, 31, 50). Nine control calves were kept at Fort Collins altitude and studied at comparable times. Calves were studied unsedated in the recumbent position. Catheters, placed by the Seldinger technique, were guided into the pulmonary artery via the jugular vein. Signals from calibrated Statham P23 Db transducers were recorded on a four-channel recorder. Cardiac output was measured by injecting cardiogreen dye into the right atrium and sampling from the descending aorta. Whereas the values are reliable for the control calves, when the high-altitude calves have a patent ductus arteriosus or an incompletely sealed foramen ovale, the values obtained may overestimate the pulmonary blood flow, as previously published (35, 50). Echocardiography was performed with a Hewlett-Packard Imager using a 3.5-MHz probe in the parasternal short- and long-axis views. After measurements had been made, the calves were euthanized by pentobarbital sodium overdose and exsanguination. The heart was immediately removed from the chest, the free wall of the right ventricle (RV) was dissected from the left ventricle and intraventricular septum (LV+S), and the weight ratio [RV/(LV+S)] was obtained as previously described (50). Ventricular tissue was processed for morphological and/or quantitative analysis as described below. The experimental protocol was approved by the University of Colorado and Colorado State University Animal Research Review Boards.

Left and right ventricular tissues from three cattle 5–8 mo old were Formalin fixed, stained with hematoxylin and eosin, and examined by light microscopy. The cattle had been born and raised at altitudes ~3,000 m and had died of pulmonary hypertension and right heart failure (1).

Tissue Samples

Fresh tissue blocks from the right and left ventricular free walls of neonatal calves were obtained from a locus between one-third and two-thirds the distance from the atrioventricular groove to the apex. Care was taken that all tissue blocks contained myocardial fibers oriented parallel to the epicardium. Tissue samples for routine histochemistry were fixed in 10% buffered Formalin (Baxter, McGaw Park, IL) for 24 h, and then placed in 70% alcohol for long-term storage. Tissue was then dehydrated and embedded in paraffin. For immunohistochemical analysis, fresh tissue was embedded in optimum cutting tissue (OCT) compound (Miles, Elkhart IN), frozen slowly in cold hexane to prevent tissue fracturing, and.
stored at $-70^\circ$C until use. Frozen OCT compound-embedded tissue was cut such that cryosections would contain longitudinal fibers at 4-$\mu$m thickness, and the sections were then applied to Suprafrost plus slides (Fisher Scientific, Pittsburgh, PA). The sections were air dried and fixed in absolute acetone for 5 min at room temperature. Fresh tissue samples for protein analysis were placed in a plastic conical tube, frozen in liquid nitrogen, and stored at $-70^\circ$C until use for protein extraction.

**Cell Morphometry**

Midventricular sections of fresh heart tissue were immediately placed into 30 mmol/l 2,3-butanedione monoxime in Krebs-Ringer solution followed by fixation in 10% buffered Formalin. Isolated cardiac myocyte morphometry measurements were performed by dissociating 1-mm sections of fixed tissue cardiac tissue samples in 50% KOH solution with gentle shaking and periodic examination under the microscope to monitor digestion. After $-2$ h, the tissue was placed in phosphate buffer solution (PBS), spun at 1,000 rpm $\times 5$ min, and decanted; the procedure was then repeated (15, 27). The isolated cells were suspended in 0.1 mol/l PBS and plated on Suprafrost slides. Myocyte length (40 myocytes/sample) was measured by light microscopy using an Olympus microscope attached to a Sony video camera and monitor, interfaced with NIH Image 1.4 software. Statistical analysis was performed using the Wilcoxon rank sum test.

**Antibodies**

Monoclonal anti-human vinculin (clone hVIN-1), monoclonal anti-$\alpha$-actin (Sarcomeric clone EA-53), anti-tubulin rabbit polyclonal, anti-pan rabbit polyclonal cadherin, and monoclonal anti-smooth muscle myosin (clone hsm-V) antibodies were purchased from Sigma Immunochimicals (St. Louis, MO). Monoclonal antibody against desmin was purchased from Biogener (San Ramon, CA). Monoclonal antibody against vimentin (clone V9) was purchased from AMAC (Westbrook, ME). Affinity-purified polyclonal anti-metavinculin antibodies and monoclonal anti-vinculin antibody (clone VII F9) were generously provided by Dr. E. Moiseeva (13) and Dr. M. Glukhova, respectively. Mouse ascites fluid (clone NS-1, Sigma, Immunochimicals) was used as an internal control for nonspecific absorption.

**Immunohistochemical, Immunofluorescence, and Light Microscopic Analysis**

Cryosections were preincubated with 5% calf serum in PBS and then incubated with primary antibodies (anti-vinculin, anti-desmin, or anti-vimentin for 1 h at room temperature and with anti-metavinculin antibodies overnight at 4°C). Antibodies against metavinuculin, desmin, and cadherin were diluted in blocking solution, 5% calf serum in PBS at 1:10, 1:100, and 1:200, respectively; anti-vinculin antibody was used as an undiluted hybridoma supernatant; and the anti-vimentin antibody was purchased in a prediluted form. Sections were processed for immunofluorescence staining utilizing a biotin-streptavidin system (biotinylated anti-mouse and anti-rabbit IgG) were purchased from Calbiochem (La Jolla, CA); streptavidin-fluorescine isothiocyanate and streptavidin-Texas Red were purchased from Amersham (Arlington Heights, IL). All reagents were applied at the dilutions recommended by the supplier. Vimentin immunohistochemistry was performed using an immunoperoxidase avidin-biotin enzyme complex as described by Hsu et al. (20). The sections were counterstained with Meyers hematoxylin (Biogenex) and embedded in Aqua-mount (Lerner Labs, Pittsburgh, PA). Routine histochemical staining with hematoxylin and eosin was used on sections to visualize alterations in cell structure. Stained sections were examined on a Nikon Optiphot-2 epifluorescence photomicroscope. Photomicrographs were taken on Kodak Ektachrome, ASA 400 film (Eastman Kodak, Rochester, NY).

**Western Blotting Assay**

For routine immunobiochemical analysis, tissue samples of freshly excised heart were frozen in liquid nitrogen. Protein extraction and SDS-PAGE (7.5% gels) were performed according to the method of Laemmli (26). Protein quantification was performed using the Bradford protein assay (6) (Bio-Rad, Hercules, CA). Protein (10 $\mu$g) was loaded onto each lane. Sarcomeric $\alpha$-actin was used as an additional loading control, because the cellular content of this protein may not be significantly affected in various forms of cardiomyopathy (30). The “soluble” (cytosolic or nonstructural supernatant) and the “insoluble” (structurally associated) fractions were analyzed for distribution of vinculin and tubulin by immediate homogenization in a stabilization buffer (50% glycerol, 5% DMSO, 10 mmol/l sodium phosphate, 0.5 mmol/l MgCl$_2$, 0.5 mmol/l EDTA, 0.5 mmol/l GTP, 100 mmol/l Trasylol, and 0.2% BSA; pH 6.95) containing a cocktail of inhibitors (10 $\mu$mol/l benzamidine, 1 mmol/l phenylmethylsulfonyl fluoride, 1 mmol/l o-phenanthroline, 10 mmol/l aprotinin, 10 mmol/l leupeptin, and 10 mmol/l pepstatin A) (19, 38), followed by high-speed centrifugation at 100,000 $g$. The supernatant and pellet from this process were solubilized in equal volumes of a 10% SDS and 10% loading buffer and then analyzed by Western blotting. Protein (30 $\mu$g) was loaded onto each lane. Equal loading of the pellet fraction was verified by Coomassie blue staining of the myosin band in the gel as previously described (30, 31).

Western blot analysis of vinculin, metavinculin, desmin, tubulin, myosin, and $\alpha$-actin in protein extracts from heart tissue was performed according to the transfer method of Towbin et al. (52). After protein transfer, the strips of nitrocellulose were incubated with antibodies against desmin (1:1,000 dilution), vinculin (1:2,000 dilution), tubulin (1:100 dilution), smooth muscle myosin (1:1,000 dilution), and sarcomeric $\alpha$-actin (1:5,000 dilution) for 1 h at room temperature. After rinsing, the blots were incubated with peroxidase-conjugated goat anti-mouse IgG (1:3,000 dilution) (Bio-Rad) for 1 h. Detection of antibody-protein complexes was performed by using enhanced chemiluminescence (Amersham) and exposing blots to autoradiography film (Kodak).

**RESULTS**

**Hemodynamics and Ventricular Weights**

**Control calves.** In 15-day-old calves born and raised at low altitude in Fort Collins, mean pulmonary arterial pressure, systemic arterial (aortic) pressure, right atrial pressure, pulmonary wedge pressure, and cardiac output were all similar to values previously reported from this laboratory (Table 1). Representative normal blood gas values in control calves studied similar to those reported previously (Table 1). The weight ratio of the free wall of the right ventricle was less than one-half that of the left ventricle plus septum (Table 1) as previously reported.
Hypoxic calves. When we interrupted the normal postnatal pulmonary circulatory transition by exposing newborn calves on the first or second day of life to continuous, severe hypoxia for 14 days, the mean pulmonary arterial pressure (as seen in Fig. 1) and right atrial pressure became elevated but aortic and pulmonary wedge pressures did not (Table 1). In three of the four calves measured, cardiac output was 4.5 l/min or less (see MATERIALS AND METHODS). There was right ventricular hypertrophy (Table 1). Echocardiography demonstrated a dilated right ventricular cavity (Fig. 2). Thus chronic, severe hypoxia had produced early right heart failure as seen by elevated right atrial pressure, increased right heart mass, and right ventricular dilation.

Routine Histology and Morphometry of Isolated Myocytes

Hematoxylin-eosin staining of the right and left ventricular tissue from control calves showed the normal pattern of relatively straight, cylindrical, parallel myocytes (Fig. 3A). The nuclei were oval and centrally located within the cell. The cytoplasm showed a normal pattern of regularly spaced cross striations. In the hypertensive failing right ventricle, the myocardium showed areas where the myocytes had a wavy, disorganized appearance and were often not in parallel orientation (Fig. 3B). In these areas, sarcomere cross striations were markedly diminished, and the nuclei were often piknotic with perinuclear vacuolization. The left ventricular tissue of hypertensive calves (data not shown).

Table 1. Measurements in control and hypoxic calves

<table>
<thead>
<tr>
<th>Variable</th>
<th>Current Measurements</th>
<th>Prior Measurements</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls</td>
<td>Hypoxic</td>
</tr>
<tr>
<td>PAorta</td>
<td>102 ± 6</td>
<td>107 ± 5</td>
</tr>
<tr>
<td>PAortic</td>
<td>(5)</td>
<td>(5)</td>
</tr>
<tr>
<td>P Right Atrium</td>
<td>12 ± 2</td>
<td>12 ± 2</td>
</tr>
<tr>
<td></td>
<td>(9)</td>
<td>(4)</td>
</tr>
<tr>
<td>PWedge</td>
<td>10 ± 1</td>
<td>12 ± 2</td>
</tr>
<tr>
<td></td>
<td>(6)</td>
<td>(4)</td>
</tr>
<tr>
<td>QPulm, l/min</td>
<td>5.1 ± 0.4</td>
<td>4.4 ± 1.0‡</td>
</tr>
<tr>
<td></td>
<td>(5)</td>
<td>(4)</td>
</tr>
<tr>
<td>RV/(LV + S)</td>
<td>0.46 ± 0.18</td>
<td>0.86 ± 0.15‡</td>
</tr>
<tr>
<td></td>
<td>(6)</td>
<td>(4)</td>
</tr>
<tr>
<td>Weight, kg†</td>
<td>41 ± 4</td>
<td>37 ± 4</td>
</tr>
<tr>
<td></td>
<td>(3)</td>
<td>(4)</td>
</tr>
<tr>
<td>PaO2</td>
<td>60 ± 2</td>
<td>24 ± 2‡</td>
</tr>
<tr>
<td></td>
<td>(4)</td>
<td>(6)</td>
</tr>
<tr>
<td>PaCO2†</td>
<td>42 ± 1</td>
<td>35 ± 2</td>
</tr>
<tr>
<td></td>
<td>(4)</td>
<td>(6)</td>
</tr>
<tr>
<td>pH‡</td>
<td>7.35 ± 0.03</td>
<td>7.38 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>(4)</td>
<td>(6)</td>
</tr>
</tbody>
</table>

Values are means ± SE. Numbers of calves are in parentheses. Values from prior measurements are taken from Refs. 10 and 50. P PulmArt, mean pulmonary arterial pressure; PAortic, systemic arterial (aortic) pressure; P Right Atrium, right atrial pressure; PWedge, pulmonary wedge pressure; QPulm, cardiac output; RV/LV + S, weight ratio of the right ventricle (RV) to the left ventricle (LV) and intraventricular septum (S); PaO2, arterial PO2; PaCO2, arterial PCO2.

*Hypoxic calves differ (P < 0.05) from controls. †Measurements were obtained in calves, both normoxic and hypoxic, of similar ages, but other than those in the current study. ‡Cardiac output values in hypoxic calves are overestimated as a result of right-to-left shunts through the foramen ovale and ductus arteriosus (see MATERIALS AND METHODS).
shown) was normal throughout and similar to that observed in the control animals.

Isolated cardiomyocytes from the failing right ventricles of hypertensive calves (104 ± 20 \( \mu \)m) were longer \((P = 0.0001)\) than those from the left ventricle (77 ± 11 \( \mu \)m) of the same animal or the right ventricle (73 ± 11 \( \mu \)m) or left ventricle (74 ± 11 \( \mu \)m) of the control calves (Fig. 4).

The failing right ventricles from cattle dying of high-altitude-induced pulmonary hypertension at age 5–8 mo had myocytes in both the left (Fig. 3C) and the right ventricles (Fig. 3D) with regularly spaced cross striations and intercalated disks. In contrast to the failing right ventricles of the neonatal calves, areas of disorganized myocardium were absent or rare in the failed right ventricles of these older cattle.

**Immunofluorescence and Immunohistochemistry**

**Vinculin and metavinculin.** Vinculin is a membrane-bound cytoskeletal protein contained within myocardial costameres and intercalated disks. Control right ventricles stained with anti-vinculin antibody demonstrated an orderly appearance of myocytes and cytoskeletal elements as previously described for normal myocardium (39). The parallel sarcomeres with their repetitive “riblike” pattern of staining (costameres) and the brighter structures at the junction of adjacent myocytes (intercalated disks) demonstrated the characteristic normal pattern of staining (Fig. 5A). In contrast, within the failing right ventricular myocardium of hypertensive calves, areas were observed that demonstrated poorly discernible patterns of costamere and intercalated disk staining with anti-vinculin antibody (Fig. 5B). In these areas, the costameres occasionally did not traverse the cell, and they gave a “broken scaffold-like” appearance. When intercalated disks could be identified, staining was diffuse and of low intensity. Compared with the control calves, the failing right ventricle had myocytes with more diffuse staining within the cell, raising the possibility of redistribu-
tion of vinculin from the contractile elements to the cytosol.

Metavinculin is a muscle-specific vinculin isoform formed by alternative splicing of the primary mRNA transcript, resulting in an additional 68 amino acid peptide inserted into the vinculin sequence (7, 24). The affinity-purified polyclonal antibodies employed in this study bind only to the unique metavinculin sequence and do not recognize vinculin (13). In control right ventricles, as also seen for vinculin, staining with the metavinculin antibody showed a normal pattern of costamere and intercalated disk structures (Fig. 5C). In the disorganized areas of the failing right ventricles, the staining with the metavinculin antibody was less intense than in the controls (Fig. 5D); there was cytoplasmic clumping of the antibody, few metavinculin-positive costameres, and irregular staining of the intercalated disks. The metavinculin staining was compatible with alteration of costamere and intercalated disk organization in these failing right ventricles and possibly with the loss of metavinculin from within the cell.

In the left ventricular tissues of both the control (Fig. 6A) and the hypertensive (Fig. 6B) animals, immunostaining for vinculin revealed an orderly appearance of myocytes and contractile elements. Thus, in the chronically hypoxic calves, the left ventricular myocytes did not show the cytoskeletal abnormalities, which were often observed in the right ventricles from the same animals.

Desmin and vimentin. Both desmin and vimentin are intermediate filament proteins that surround each myofibril Z disk and form networks within each Z plane of the muscle fiber (18). Desmin is a protein essential to the normal linkage of intermediate filaments with the sarcolemma (14, 53). In control right ventricles of the present study, desmin antibody staining revealed an orderly appearance of costameres and intercalated disks (Fig. 7A). In contrast, myocyte costameres and intercalated disks had little or no staining for desmin in focal areas within the failing right ventricles (Fig. 7B), but rather staining was diffuse within the cell, suggesting redistribution from the cytoskeleton to the cytosol. In left ventricles (not shown) of both hypoxic and normoxic calves, staining was normal. Thus cytoskeletal disorganization, as seen by staining for desmin, selectively involved the failing right ventricle.
Vimentin is present in several cell types within cardiac tissue (49) and may be a more important component of the myocyte during development than in the adult (36, 54). In the present study, immunostaining of control right ventricles with anti-vimentin antibodies demonstrated a distinct pattern of regularly spaced costameres (Fig. 7C). No staining of the intercalated disks was identified. Staining of cells other than myocytes (probably fibroblasts) was observed. In hypertensive failing right ventricles, staining of vimentin with the vimentin antibody was almost absent (Fig. 7D), and there was no apparent costamere staining. Vimentin staining was primarily observed in cells (probably fibroblasts) other than myocytes. The pattern of vimentin staining in left ventricular myocytes from pulmonary hypertensive calves was normal and similar to that observed in the right and left ventricles of control animals.

Cadherin. Cadherin is a calcium-dependent cell adhesion molecule that is predominantly associated with intermyocyte junctions (the intercalated disks) and is thought to be essential for the integrity of intercellular connections and cardiac cell compartmentalization (17, 29). In the present study, immunostaining of myocardial tissue for cadherin demonstrated normal intercalated disk appearance in the right ventricles of control animals (Fig. 7E). In some areas within the failing right ventricle, intercalated disk structure was severely disrupted (Fig. 7F), but no abnormalities of disk structure were noted in the left ventricle (not shown) from control or pulmonary hypertensive animals.

Western Blot Assays

Western blot analyses were performed to assess possible differences in the quantity and distribution of protein expression in randomly selected portions of right ventricular muscle. In the control right ventricle, Western blot analysis indicated that vinculin was the major isoform expressed with less abundance of the metavinculin protein isoform (Fig. 8A). In the failing right ventricle, Western blot analysis did not show any differences in the relative abundance of vinculin compared with control. However, metavinculin protein in the failing right ventricles was barely detectable (Fig. 8A). In left ventricles, Western blot analysis for vinculin and metavinculin showed no differences between the control and high-altitude animals (data not shown).

Little or no difference in desmin or α-actinin abundance was found on comparing the failing with the control right ventricle (Fig. 8B) or on comparing the control and high-altitude left ventricles (data not shown).

Crude separation of cell fractions from the control and failing right ventricles showed differences in the distribution of free and structural forms of vinculin. Compared with controls, there was a relative redistribution of vinculin from the structural (insoluble pellet) myocyte fraction to the free cytosolic (supernatant) fraction in the failing right ventricle (Fig. 9). These findings were consistent with the immunofluorescent observations (Fig. 5) in the Immunofluorescence and Immunohistochemistry section, where vinculin localization to specific cellular structures was reduced in the failing right ventricles.

In the right ventricular myocardium from control calves, cadherin was identified in the structural (insoluble pellet) and not in the free cytosolic (supernatant) fraction (Fig. 9). Neither the content nor distribution between the insoluble and soluble fractions appeared different from the control in the failing right ventricular myocardium. In the right ventricular myocardium from control calves, tubulin was present in both the soluble and insoluble fractions, with a greater content in the latter than the former (Fig. 9). In the right ventricular myocardium from the calves with failure, the tubulin content in the insoluble fraction was greater than in controls, whereas the content in the soluble fraction was less than control.

DISCUSSION

The main finding of the present study was that within the hypertensive and failing right ventricle of hypoxic, neonatal calves, there were areas showing marked disruption of the normal myocardial cytoskeleton. The disruption was seen on standard light microscopy as areas with decreased or absent cross striations. Striations represent Z lines and the overlap of
contractile proteins and the intercalated disks where cytostructural proteins normally attach. Loss of these striations suggested that the associated structural proteins had become disorganized. Confirmation of disorganization of the cytostructural proteins was seen by immunohistochemical analyses, which showed areas with markedly altered patterns of expression of vinculin, metavinculin, desmin, and vimentin. The findings were consistent with disruption, decrease, and/or loss of the intermediate proteins within costameres and/or intercalated disks. Thus, in the hypoxic failing neonatal right ventricular myocardium, there was both a loss

Fig. 7. Immunostaining for desmin, vimentin, and cadherin in RV myocardium. A: immunostaining with an anti-desmin antibody in a control calf showed normal cross striations of costameres (straight arrows) and intensely stained intercalated disks (curved arrow). B: in a chronically hypoxic hypertensive calf, there was a loss of staining for desmin in costameres and intercalated disks, and desmin was distributed diffusely through the myocytes. C: immunostaining for vimentin in a control calf showed normal cross striations of costameres. D: in a chronically hypoxic hypertensive calf, there was a loss of staining for vimentin in myocytes. *Nonmyocytes, probably fibroblasts. E: staining for cadherin in a control calf showed intercalated disk structures. F: in a chronically hypoxic hypertensive calf, cadherin was retained within myocytes, but intercalated disk structures could not be identified (bar = 10 µm).
of cross striations and disarray of the cytoskeletal architecture, suggesting that myocytes with a loss of cross striations also had the associated cytoskeletal disorganization.

In the failing right ventricle, when vinculin was not readily identified by immunohistology in the costameres and intercalated disks of the myocyte, it appeared to be diffusely distributed within the cytosol, a suggestion supported by Western blot analysis of random tissue samples. This analysis suggested that in right heart failure the total content of vinculin had not decreased, but that it was now largely in the cytosolic, rather than the structural, fraction of the myocardial sample. Both Western blot and immunohistological analyses were compatible with the conclusion that vinculin had dissociated from the contractile apparatus and was redistributed within the cytoplasm. Whereas the mechanism remains obscure, the binding interaction of vinculin with actin microfilaments (22) and the cell membrane is regulated by phosphatidylinositol-4,5-bisphosphate, a pathway induced by the GTP binding protein Rho (16). Possible disruption of this pathway in neonatal heart failure deserves further study.

Some dissociation of vinculin from the intercalated disks with redistribution into the periphery of the cell has been shown to occur with left heart failure in young guinea pigs (57, 58). These authors found that the amount of desmin in the intercalated disks increased and, in contrast to our findings, that some vinculin and desmin remained in the costameres. The two animal models of failure were quite different. The aorta was banded at 1 mo in guinea pigs, which produced left heart failure in 6 mo, whereas the calf was placed at high altitude shortly after birth, which produced right heart failure in 2 wk. However, a common thread may be that when vinculin and desmin dissociate from the contractile apparatus, they become redistributed within the myocyte. For example, when the myocyte is undergoing cell division, the contractile apparatus is completely disassembled, and vinculin and desmin are retained diffusely within the cytosol (11). An analogous process appeared to occur in some myocytes in neonatal right heart failure. In our calves the failure is promptly reversible by returning the animal to low altitude. Possibly, study of this model may provide insight into the mechanisms of stability of the myocyte cytoskeleton, its assembly, its disassembly, and, with recovery from failure, its reassembly.

Neonatal right heart failure altered the other cytoskeletal proteins, but patterns of alteration differed from that of vinculin and desmin. Metavinculin, an important structural protein in the adult heart (3, 24, 30), was normally present in the costameres and intercalated disks of the neonatal calf heart. Like vinculin, metavinculin dissociated from the contractile elements in neonatal heart failure; but unlike vinculin, the content of metavinculin within myocytes decreased, and that which remained in the cytosol was in clumps and
not evenly distributed. Vimentin, which is absent or minimally present in the adult cardiac myocyte of some species (45, 54), was present in the normal neonatal calf hearts. However, when there was right heart failure, and in contrast to vinculin and desmin, the content of vimentin within the myocyte markedly decreased. Cadherin, an essential normal component of intercalated disks (17, 29–31), was retained in the myocyte and even in the structural fraction in the failing right ventricle. However, its appearance by immunohistology indicated severe disruption of the intercalated disks. The high content of tubulin in the failing right heart was expected from published reports, which have indicated that it contributes to impairment of ventricular contraction (51, 56). These differences between the failing and nonfailing ventricles, not only for vinculin and desmin, but also for metavinculin, vimentin, and tubulin, probably reflect substantial changes in the cytoskeletal architecture and function with neonatal heart failure, the significance of which will require future investigation.

Patchy areas of disordered myocardial cytoskeleton have been reported in other forms of heart failure. In end-stage human dilated cardiomyopathy, Schaper et al. (45) reported loss of contractile elements and disorganization of the myocyte cytoskeletal proteins, including desmin and vinculin. Dogs with chronic heart failure from repeated coronary embolization have also been found to have areas with severe disruption of sarcomeres and partial loss of Z bands (47). The etiologies of the heart failure in humans and dogs differ from that in our neonatal calves, but the findings in each are consistent with the development of patchy cytoskeletal disarray in heart failure.

The findings of the present study must be considered in the context of when during development we imposed on the right ventricle the dual challenges of hypoxia and high pressure. Previous studies (10, 35, 41, 50) would suggest that because our calves were born and lived for a day or two in a normoxic environment, the right ventricular pressures likely fell well below fetal values before pulmonary hypertension developed in the hypoxic chamber (Fig. 1). We do not know whether this transient fall in right ventricular pressure adversely affected the ability of the right ventricle to handle a subsequent pressure load. By comparison with our calves in neonatal heart failure, children born with large ventricular septal defects and obstruction to outflow of the right ventricle (tetralogy of Fallot) also have arterial hypoxemia and systemic pressures in the right ventricle. But unlike the calves, the children rarely have right heart failure or ventricular degeneration until they are several years of age (23). In these children, it is possible that ventricular adaptation before birth delayed their development of right heart failure.

The results in our calves also raised important issues relating to the magnitude and time course of the imposed pressure load and also the severity of the associated hypoxemia. Cattle born into the more moderate altitude of −3,000 m (vs. 4,570 m in the present study) or brought there as adolescents develop pulmonary hypertension over many weeks, months, or even years (1, 42, 60, 61). Not only might the right ventricle have more time to adapt, but also the lower altitude would mean less severe hypoxemia. But in the present study, when systolic pressure in the right ventricle approached that in the left ventricle, myocardial perfusion was likely limited primarily to diastole, raising the possibility that coronary flow was impaired. And when, in addition, there was profound hypoxemia at altitude, one must consider that right ventricular oxygenation might not keep up with demand, leading to myocardial ischemia, an injury which would contribute to heart failure (8). In any event, when cattle have had right heart failure from slowly developing pulmonary hypertension at lower altitudes, the right ventricular myocytes have not shown the cytoskeletal disorganization that was observed in the hypoxic neonatal calves of the present study.

But why did the left ventricle not fail? As implied above, the partial pressure of oxygen of the coronary arterial perfusate would be the same for both ventricles, and, at the time of right ventricular failure, right and left ventricles had similar afterloads. Yet despite these factors, the right but not the left ventricle failed, as evidenced by the high right atrial but normal wedge pressures, the echocardiograms showing dilation in the right and not the left ventricular cavity, and elongation of the isolated myocytes from the right, but not the left, ventricle. The present study cannot establish the reasons for the selectivity of right heart failure. However, we can refer to inherent differences in structure and function of the two ventricles (34, 44) and acknowledge the differences in coronary supply and the fact that the pressure would have transiently decreased after birth in the right but not the left ventricle. Whatever the reasons, the right and not the left ventricle failed, and only right ventricular myocytes showed cytoskeletal disorganization.

The present study raises the possibility that the neonatal right ventricle, undergoing its normal pressure fall and changing myocyte architecture after birth, may be particularly susceptible to chronic pressure overload in the presence of hypoxemia. If so, one expects that imposing a load on the fully developed, mature myocardium would be better tolerated than in the neonatal period. The evidence is incomplete, however, and studies are needed that compare, for example, overloading the right ventricle of the newborn calf with an overload imposed on the right ventricle of older cattle. Establishing whether there are unique myocyte cytoskeletal changes with heart failure in the neonatal period has important consequences for understanding the postnatal development of the myocardium and for providing guidance to physicians who care for newborn children.

We appreciated the helpful advice from B. Perryman and A. Robinson. Sections of right and left ventricles from cattle dying of “brisket disease” were kindly provided by Dr. D. Gould, Professor of Pathology at the School of Veterinary Medicine, Colorado State University, Fort Collins.
REFERENCES


