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Deficiency of Actinin-Associated LIM Protein Alters Regional Right Ventricular Function and Hypertrophic Remodeling

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Abstract

Targeted deletion of actinin-associated LIM protein (ALP) in mice leads to right ventricular (RV) dysplasia and a mild RV cardiomyopathy. Although the phenotype has been thoroughly characterized, the mechanisms leading from the cytoskeletal defect to the disease are unclear. We hypothesized that ALP deficiency may be associated with (1) changes in regional systolic dysfunction and (2) regional dysregulation of hypertrophic growth, in accordance with the restricted expression of ALP in the outflow tract of the RV. We examined RV regional epicardial systolic strains with respect to end-diastole in ALP knockout (ALPKO) mice and wild-type controls using an open-chest preparation. Strain components were consistently lower in the ALPKO mice than wild-type controls (second principal strain E_2 : $p = 0.05$). RV pressure was slightly but not significantly lower in ALPKO mice as well. To assess regional growth, geometric remodeling was analyzed in ALPKO and wild-type mice after 4 weeks of chronic hypoxia (11% oxygen). The average amount of RV wall thickening in response to hypoxia was reduced to 11% in the ALPKO mice compared with 44% in the wild-type controls. In summary, the results are consistent with the view that disruption of ALP is associated with diminished RV contractile function as well as altered hypertrophic remodeling.

Keywords

cardiac function; mice; cytoskeleton; cardiomyopathy; hypoxia

INTRODUCTION

The cytoskeletal protein ALP (actinin-associated LIM protein) binds to α -actinin with high affinity and is mostly concentrated at the intercalated disc in cardiac muscle. It is expressed in the mouse heart symmetrically up to embryonic day 7.5, but is restricted to the infundibular region of the RV and parts of the septum after birth.²⁰ Homozygous deletion of ALP in mice results in primarily right ventricular (RV) dysmorphogenesis, a decrease in trabeculation and mild dilated cardiomyopathy (DCM),²⁰ resembling human arrhythmogenic RV dysplasia (ARVD).^{4,9} Thus, the phenotype appears to correlate with the expression of ALP in the adult heart. However, further functional information and data regarding the mechanisms leading to the disease are unavailable.

The association of ALP with plakoglobin,²⁰ an important anchoring protein at the intercalated disc,^{5,16} which has itself been linked to ARVD,¹⁷ suggests that ALP disruption may result in a decrease in cardiac myocyte stability and possibly impaired force transmission. This idea is further supported by the finding that ALP contains one LIM and one PDZ domain, both of which are particularly known for protein–protein interaction. ALP deficiency could thus cause instability in the intercalated disc region and lead to impaired systolic function.

ALP may also be involved in mechanical signaling in the cardiac myocyte and thus growth and remodeling processes. The finding that ALP possesses one LIM and one PDZ domain points to this possibility, since some LIM domain proteins are known to have intracellular signaling functions [reviewed in Brown *et al.* (2001)²] including mechanotransduction,¹³ and since PDZ domain proteins are particularly known for being involved in the assembly of signaling complexes [reviewed in Fan and Zhang (2000)⁸]. Moreover, ALP is concentrated at the intercalated disc, which is believed to be an important structure in myocyte signal transduction.^{10, 22}

To test the hypothesis that RV DCM observed in ALP deficiency is associated with impaired regional systolic performance, we examined RV regional epicardial systolic strains in two dimensions as well as RV pressure in ALP knockout (ALPKO) and wild-type mice. The sinus region (or inflow region) and the infundibulum (or outflow tract) form from distinct cell populations in the embryonic heart.^{14,25,28} The circumstance that ALP is restricted to the infundibular region in the adult heart, but is expressed ubiquitously in the fetal state, provides the opportunity to study the regional effects of ALP. The goal of this study was to determine whether disruption of ALP is associated with (a) attenuated systolic function in the RV, (b) abnormal hypertrophic remodeling in response to hypoxia, or both. We determined further, whether these changes occur only in the infundibular region and thus may result from the immediate absence of ALP, or whether they occur independent of region and thus may result from a lasting effect of ALP everywhere in the embryonic myocardium.

METHODS

RV Strain Measurements

ALPKO mice ($n = 10$) and wild-type controls ($n = 13$) were anesthetized using a ketamine/xylazine mixture (ketamine 100 mg kg⁻¹, xylazine 8 mg kg⁻¹). A tracheotomy was performed, and the mouse was ventilated with room air with a tidal volume of 0.5 ml at a rate of 100 strokes min⁻¹. The chest was opened and the pericardium removed. Titanium dioxide markers were arrayed on the surface of the RV free wall to visualize wall deformations, a technique previously used to assess regional systolic function in the isolated murine heart.¹¹ The markers were videotaped using a CCD camera (COHU Inc.) while ventilation was temporarily switched off, in order to minimize rigid body motion of the heart.

The videotaped frames were split into fields using Scion Image (Version Beta 4.0.2) to increase temporal resolution to 60 Hz. Homogeneous two-dimensional epicardial strains in

both regions were computed from three markers each (Fig. 1) under the assumption that the plane of the markers was oriented perpendicular to the axis of the camera. Strain values at the time of maximum RV pressure (see below) were compared between the genotype groups.

Measurement of RV Pressure

On completion of strain recordings, a 27-gauge needle, connected to a fluid-filled disposable pressure transducer, was inserted into the RV by direct puncture of the RV free wall close to the apex.^{15,19,26} Maximum developed pressure was measured and compared between the groups.

Staining for LacZ Expression

ALPKO hearts were stained for expression of the *lacZ* reporter gene, which had been inserted in to the locus of ALP. In five of the ALPKO mice used for strain measurements, the heart was excised and retrogradely perfused for several minutes in arrest solution (containing heparin 10,000 units l⁻¹ and in mmol l⁻¹: NaCl 68, KCl 30, NaHCO₃ 36, MgCl₂·6H₂O 1, Na₂SO₄ 2, dextrose 11 and 2,3-butanedione-monoxime 30). Both ventricles were kept slightly inflated using blunt needles connected to a pressure head in order to prevent the RV from collapsing or buckling. Subsequently, the heart was perfused with fixative containing 5% glutaraldehyde for at least 30 min at a constant pressure of approximately 60 mmHg and quickly frozen in OCT Tissue-Tek Compound (Sakura Finetek USA Inc.) using dry ice. Cryosections of 12 µm were taken throughout the heart, permeabilized in phosphate buffered saline (PBS) with 0.02% sodium deoxycholate and 0.01% NP-40 for 1.5 h and incubated in X-Gal solution (in mmol l⁻¹: X-Gal 2.5, K-ferrocyanide 5.0, K-ferricyanide 5.0, and MgCl₂ 2.0 in PBS) at 37°C for 24 h. These sections and additional sections obtained in the same way from three wild-type hearts were subsequently stained with eosin.

Hypoxia-Induced RV Hypertrophy

Chronic hypoxia for several weeks is often used to induce RV hypertrophy.^{7,12,24,26,29} For this study, 12 ALPKO mice and 16 wild-type mice of the same strain were randomly divided into two groups. Seven ALPKO and eight wild-type mice were housed in a normobaric environmental chamber for 4 weeks with an oxygen concentration of 11%. The remaining mice were breathing room air. All mice were exposed to a 12-h light–dark cycle and received food and water *ad libitum*. After 4 weeks of hypoxia, the ALPKO mice underwent RV pressure measurements and their hearts were sectioned longitudinally and stained for *lacZ* expression as described above.

All experiments were performed under protocols approved by the Animal Subjects Committee, University of California, San Diego.

Regional RV Hypertrophy Index

To quantify the difference in amount of hypertrophy between the sinus and the infundibular regions, taking into account all sections, a regional hypertrophy index was formulated.

$$K_{RV} = \frac{V_{lacZ}}{\sum_{i=1}^n (A_{lacZ}/A_{tot})_i V_i}$$

A_{lacZ} and A_{tot} represent the *lacZ*-expressing and total areas of the RV in one section, respectively, and are calculated as the product of the RV length (Fig. 2) and the distance to the next section. Similarly, V_i is the RV volume in the same section. V_{lacZ} represents the total *lacZ*-expressing RV volume in the entire heart. The index therefore quantifies a ratio of lengths, which corresponds to the relative thickness of the *lacZ*-expressing and non-*lacZ*-expressing regions. If $K_{RV} > 1$, the *lacZ*-expressing region is relatively thicker than the non-*lacZ*-expressing region. Conversely, if $K_{RV} < 1$, the non-*lacZ*-expressing region is thicker.

Statistical Analysis

Statistical comparisons were made using repeated measures ANOVA for strain measurements and Student's *t* test for all other comparisons. The *p* values of 0.05 and below were considered statistically significant. All data are expressed as mean $\pm$ SEM.

RESULTS

Regional Epicardial RV Strains

Two representative cycles of the first principal strain E_1 with respect to end-diastole in an ALP knockout mouse are shown in Fig. 3. Maximum strain was greater in the infundibular region than the sinus region. The results of the average individual strains, selected at the time of maximum pressure, are summarized in Fig. 4, where E_{11} is the circumferential strain, E_{22} the longitudinal strain, E_{12} the shear strain, and E_1 is the first principal strain and E_2 the second principal strain, α the principal angle. The corresponding *p* values from repeated measures ANOVA are shown in Table 1. Strains were lower in the sinus than in the outflow tract region in both genotypes, which reached statistical significance in the case of E_{22} . In terms of the differences depending on genotype, strain components were generally lower in the knockout hearts compared with wild-type controls in both regions. This difference was particularly noticeable in the case of E_{22} and E_2 but did not reach statistical significance, if each strain component was evaluated separately with repeated measures ANOVA. However, the areal stretch, computed as the product of the two principal stretch ratios averaged in both regions, was significantly lower in the ALPKO mice than controls (ALPKO: $20.5 \pm 2.3\%$ vs. WT: $26.2 \pm 1.5\%$, $p = 0.03$). In contrast, none of the strain components produced a significant interaction effect (interaction *p* value, Table 1). These values by themselves would indicate that strains were not affected differently by region. However, the high individual variation of regional ALP/*lacZ* expression suggested the possibility that the regions of infundibular and sinus strain measurement did not coincide ideally with the regions of high and low ALP/*lacZ* expression, respectively.

Correlation Between Strains and ALP (*lacZ*) Expression

To further investigate the strain differences depending on region, we compared strains (at the time of maximum first principal strain) based on the difference of *lacZ* expression in the respective area in five ALPKO hearts. *LacZ*-stained sections from a wild-type and a

knockout heart are illustrated in Fig. 5, showing the areas where ALP would have been expressed in dark blue. After the information on *lacZ* expression area from all longitudinal sections through the heart was mapped onto the video image, the fractional area of *lacZ* expression within the triangles of strain measurement was rated between 0 (no expression) and 1 (maximum expression). The strain components measured in the knockout hearts are plotted against the difference of *lacZ* expression between the regions in Fig. 6(A). Despite a large variation, a trend was observed that strains increased in magnitude with higher differences in *lacZ* expression. To test this possibility further, ALPKO mice were divided into two groups according to the difference in *lacZ* expression (above and below 0.7). The difference in strain between the regions was plotted for these two groups as well as all five ALPKO together and wild-type controls [Fig. 6(B)]. The ALPKO group with a high difference in *lacZ* expression between the regions showed the highest change in strains compared with the wild-types (E_{11} : $p = 0.04$, E_1 : $p = 0.02$), whereas the ALPKO group with low difference in *lacZ* expression showed strains most similar to the wild-types. These results suggest that strains are particularly decreased in the infundibular region in the ALPKO, pointing to a direct effect of ALP deficiency on systolic function.

RV Pressure

As another indicator of contractile function in ALPKO myocardium and overall ventricular performance, RV pressure was measured. Three ALPKO hearts and two wild-type hearts were excluded from pressure analysis due to arrhythmias, pulsus alternans, or excessive damage to the RV free wall. Developed RV systolic pressure was slightly but not significantly higher in the wild-types than the ALPKO RV (10.7 ± 5.0 mmHg vs. 9.9 ± 3.6 mmHg, $p = 0.70$).

ALPKO Heart Weight and RV Pressure After Hypoxia

Table 2 summarizes heart weight, heart rate, and RV pressure (during anesthesia) in ALPKO mice after hypoxia compared with the normoxic control group. Unexpectedly, the heart weight was somewhat lower in the hypoxic group, although this was not statistically significant. Heart rate during the pressure measurements was similar in both groups. The difference between systolic and diastolic pressure was significantly elevated in the hypoxic group, confirming pulmonary hypertension due to hypoxia.

RV Wall Remodeling in Response to Hypoxia

In wild-type mice exposed to chronic hypoxia for 4 weeks, the normalized RV thickness increased by nearly 50% [Fig. 7(A), $p = 0.02$], which confirms RV hypertrophy and is consistent with the typical RV weight increase seen after 3–4 weeks of hypoxia.^{1,12,29} In this study, it was not feasible to weigh the ventricles separately, because we were interested in regional hypertrophy in the RV. Instead, the entire hearts were sectioned longitudinally in mice from the hypoxic ($n = 6$) and normoxic ($n = 5$) groups and stained for *lacZ* expression. To minimize possible variation in the data due to partial contracture of the heart after the time of arrest, the RV free wall thickness was normalized by LV thickness. In contrast to the wild-type mice, ALPKO mice showed only an 11% increase in normalized RV thickness in response to hypoxia [Fig. 7(A)] which was not significant ($p = 0.4$). This leaves the

possibility that the *lacZ*-expressing region responded differently to hypoxia than the non-*lacZ*-expressing region. The hypertrophy index K_{RV} indicates the average regional hypertrophy and takes into account regional thickness in all sections of ALPKO mice [Fig. 7(B)]. K_{RV} was not significantly different between normoxia and hypoxia ($p > 0.7$), suggesting that the amount of hypertrophy in ALPKO mice was similarly blunted in both regions. Interestingly, K_{RV} was smaller than 1 in both groups, indicating a slightly thinner *lacZ*-expressing (normally ALP-expressing) region in all ALPKO.

DISCUSSION

We investigated the hypotheses that ALP deficiency with the known RV dysmorphia and mild DCM²⁰ is associated with (1) impaired regional systolic function of the RV and/or (2) dysregulated regional RV hypertrophy. The hypotheses were tested by measuring regional epicardial systolic RV strains and analyzing regional hypertrophy after chronic hypoxia, respectively.

Regional Systolic Function

Systolic strains were lower in the ALPKO than the wild-type in both regions, suggesting that ALP deficiency may not only affect systolic performance in the outflow tract, where ALP would normally have been expressed, but also may leave a long-term effect on systolic RV function in the sinus region, where ALP would have been expressed only during the fetal stage. The decreased areal stretch in the ALPKO RV also confirms that ALP deficiency may have an indirect effect on contractile function. RV pressure was only slightly lower in the ALPKO than the wild-type control, which may reflect the diminished systolic strains in ALP deficiency. Due to the small difference in strains between the genotypes, it is not surprising that RV pressure was relatively similar in both groups.

Additionally, we observed the tendency that the difference in strains between the regions was higher in knockout mice with a high difference in *lacZ* expression than in knockout mice with low difference in *lacZ* expression. In other words, there was a correlation between the regional difference in strain and the regional difference in *lacZ* expression. This suggests that the immediate presence of ALP may be required for adequate systolic function in the RV outflow tract. On the basis of the ALP expression pattern, it would then be expected that the sinus strains are unaffected, whereas infundibular strains are directly influenced by ALP deficiency. We found, on the contrary, that strains were affected more in the sinus region than in the infundibular region. This may be explained by the tight mechanical coupling and mutual interaction of the two regions. Taken together, our results suggest that systolic strains in the RV of ALPKO mice may be directly and indirectly influenced by ALP disruption.

The amount of maximum strain measured (on average about 17%) was similar to previously measured RV strains in the conscious dog,^{3,18,23} despite the substantially slowed heart rate in the anesthetized mouse in this study (250 bpm) compared with a conscious mouse (500–600 bpm). The literature contains conflicting results regarding regional strain differences. We found that strains were somewhat lower in the sinus region than the outflow tract region, which is consistent with some previous studies,^{3,21} but disagrees with others.^{18,27} The reasons for these discrepancies are not clear, but are likely due to differences in preparation

such as in marker placement and open- vs. closed-chest measurements. RV pressure has been measured frequently in mice using similar methods in recent years, and developed pressures ranged between 8 and 30 mmHg,^{6,29} possibly depending on the type and amount of anesthetic used. Our RV pressure data, therefore, also agree with the literature.

In summary, although overall systolic RV function was not diminished, some changes in strains were found in the absence of ALP, indicating that ALP may play a role in active force transmission or contractile regulation. Specifically, the data suggest that ALP expression may both directly and indirectly influence RV systolic function. ALP expression may thus be required everywhere in the myocardium in the fetal stage as well as in the outflow tract in the adult for proper systolic performance of the right ventricle.

Regional Hypertrophy

We analyzed RV wall geometry after 4 weeks of hypoxia in wild-type mice and additionally measured RV pressure in ALPKO mice, to test the hypothesis that the RV DCM observed in ALPKO mice is associated with impaired hypertrophic remodeling.

RV pressure was elevated in the hypoxic group compared with the normoxic ALPKO mice, while heart rate was not altered. The pressure increase is consistent with the expected pulmonary hypertension as a result of chronic hypoxia and is comparable to that reported in the literature.^{7,12,24}

The wild-type mice showed a significant overall increase in RV thickness of 44% in response to hypoxia, which is in good agreement with reports of RV weight change after similar hypoxia treatment.^{1,12,29} RV thickening was substantially attenuated in ALPKO mice, however, and was even accompanied by a decrease in heart weight (Table 1). This result suggests that ALP deficiency may lead to insufficient geometric ventricular remodeling in response to a growth stimulus. In order to elucidate the question of regional remodeling based on *lacZ* expression in the ALPKO and to include the thickness and *lacZ* expression information from all sections, a regional RV hypertrophy index was formulated, which expresses the relative thickness of the *lacZ*-expressing and the non-*lacZ*-expressing regions. We observed a slightly thinner *lacZ*-expressing region in both groups. If hypertrophy was impeded only in the RV outflow tract as a result of ALP deficiency, the hypoxic group would be expected to show a considerably smaller RV index than the normoxic group. Our finding that the index did not change after hypoxia suggests that the observed decrease in RV overall hypertrophy was even over the entire ventricle and did not correlate with ALP expression in the adult. Therefore, it is likely that ALP disruption during the fetal stage was responsible for the difference in hypertrophic growth in the adult animal.

Limitations

A major limitation of this study was that the penetrance of the ALP deficiency is very low, such that RV dilation and cardiomyopathy only occur in 20–30% of ALPKO mice. Proteins such as RIL or CLP-36, which are highly homologous to ALP, may compensate for the lack of ALP by partially taking over its function, thus leading to the low penetrance.²⁰ In this study, mice were not selected on the basis of phenotype but on genotype, leaving the

possibility that only 2–3 of the 10 ALPKO mice showed cardiomyopathy. This may explain the large variation as well as the small differences in strain. Finally, since the heart contracted on its own sinus rhythm during strain and pressure measurements, differences in heart rate occurred, probably influencing the strain data. However, these influences are likely systematic and probably did not have a significant effect on the regional comparisons.

Summary

The results show that regional systolic RV strains are decreased in ALP deficiency. Additionally, hypoxia-induced hypertrophic remodeling appeared impaired in ALPKO mice. Therefore, our results suggest that ALP disruption is associated with a regional impairment of systolic function as well as an abnormality in hypertrophy and growth regulation in the RV.

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FIGURE 1.

Camera view of the right ventricle during strain measurements, showing the white titanium dioxide markers attached to the epicardium. Three of these markers were used in each region (triangles) for homogeneous systolic strain measurements. RA: right atrium, RV: right ventricle, LV: left ventricle.

FIGURE 2. Longitudinal section of an ALPKO heart stained for *lacZ* expression (dark areas), indicating how the lengths of the RV (*lacZ*-expressing, L_{lacZ} , and total, L_{tot}) were determined. The lengths were measured in the center of the wall.

FIGURE 3.

Representative time course of the first principal strain in infundibulum and sinus regions in an ALPKO RV in two contraction cycles.

FIGURE 4.

RV systolic strains in outflow tract and sinus regions at the time of maximum RV pressure.

E₁₁: circumferential strain, E₂₂: longitudinal strain, E₁₂: shear strain, E₁ and E₂: principal strains, α : principal angle.

FIGURE 5.

Examples of *lacZ*- and eosin-stained longitudinal sections from a wild-type and an ALPKO heart, showing RV geometry and the region where ALP would have been expressed (dark blue). Slight dilation of the RV in the ALPKO is visible.

FIGURE 6.

Relationship between strains and *lacZ* expression. (A) Strain difference between the regions plotted against the rated difference in *lacZ* expression in five ALPKO hearts. (B) Strain difference between the regions expressed after grouping ALPKO hearts based on the difference of *lacZ* expression. The asterisks indicate a significant difference ($p < 0.05$) between the results in the respective column and the wild-type column.

FIGURE 7.

Hypertrophic remodeling in response to chronic hypoxia. (A) Normalized RV free wall thickness (RV/LV thickness) in response to hypoxia measured in the center longitudinal section from ALPKO and wild-type hearts. * $p < 0.05$, significant difference from normoxia. (B) RV hypertrophic index as a measure of relative thickness of *lacZ*-expressing and non-*lacZ*-expressing regions in ALPKO hearts. Since the index is smaller than 1, the non-*lacZ*-expressing region was slightly thicker than the *lacZ*-expressing region in both groups. Hypoxia did not produce a significant change in this index ($p > 0.7$).

TABLE 1
Repeated measures ANOVA *p* values of strain components analyzed at the time of maximum pressure.

	E₁₁	E₂₂	E₁₂	E₁	E₂	α
Genotype	0.38	0.07	0.78	0.42	0.05 ^{ns}	0.50
Region	0.54	0.001 [*]	0.12	0.22	0.28	0.02
Interaction	0.08	0.31	0.16	0.30	0.11	0.34

TABLE 2

Heart weight (HW), heart rate (HR), and RV pressure difference in normoxic and hypoxic ALPKO mice.

	HW (mg)	HR (bpm)	Pressure difference (mmHg)
Hypoxia	147 ± 11	280 ± 12	34.2 ± 2.5
Normoxia	166 ± 14	273 ± 23	22.0 ± 3.8
<i>p</i> value	0.3	0.8	0.03*