Maturation-Dependent Differences in Regulation of Sarcoplasmic Reticulum Ca^{2+} ATPase in Sheep Myocardium in Response to Pressure Overload: A Possible Mechanism for Maturation-Dependent Systolic and Diastolic Dysfunction

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ABSTRACT

We have previously demonstrated that pressure-overload hypertrophy in adult sheep is associated with myocardial dysfunction whereas that in young lambs is associated with normal contractility. To probe for possible mechanisms of these age-dependent differences, we assessed mRNA expression of genes encoding critical components of myocardial Ca^{2+} handling in the same animal model. We studied left ventricular myocardium of young and adult sheep with short-term (48 h) and long-term (6 wk) pressure overload induced by ascending aortic constriction. Six weeks of pressure overload induced the significant left ventricular hypertrophy (36 and 39% increase in left ventricular/body weight ratio in lambs and sheep, respectively). The Ca^{2+} ATPase and $\text{Na}^+/\text{Ca}^{2+}$ exchanger mRNA decreased with pressure overload only in the adult ($p < 0.05$). Ca^{2+} channel mRNA was slightly increased by pressure overload regardless of age ($p < 0.05$). Calsequestrin, sarcoplasmic reticulum Ca^{2+} release channel, or myosin heavy-chain mRNA levels did not significantly differ. In adult sheep after 6 wk of pressure overload, decreases in load-adjusted midwall shortening (systolic dysfunction)

and prolongation of relaxation time constant (diastolic dysfunction) correlated with decreases in Ca^{2+} -ATPase mRNA. The sarcoplasmic reticulum Ca^{2+} -ATPase protein level and Ca^{2+} uptake activity of isolated sarcoplasmic reticulum vesicles were depressed only in the adult with pressure-overload hypertrophy but not in the young. We demonstrated age-dependent differences in mRNA expression of Ca^{2+} -handling protein genes in response to pressure overload, which preceded the occurrence of hypertrophy and myocardial dysfunction. Thus, altered expression of Ca^{2+} -handling protein genes may be one of the primary responses to pressure overload rather than a phenomenon secondary to myocardial hypertrophy. (*Pediatr Res* 50: 246–253, 2001)

Abbreviations

LV, left ventricle
DHP, dihydropyridine
SR, sarcoplasmic reticulum

LV pressure-overload hypertrophy is often accompanied by myocardial dysfunction. Several factors such as severity, duration, and abruptness of pressure overload determine myocardial dysfunction. Maturation is also a factor that determines myocardial adaptation to pressure overload (1, 2). We have previously shown that myocardial systolic function (3) and diastolic function reserve (4) are depressed in conscious adult sheep with LV hypertrophy. Interestingly, young animals with

a similar degree of pressure-overload hypertrophy maintain normal systolic and diastolic function (3, 4). Thus, there is an age-related difference in myocardial adaptation to pressure overload. Although mechanisms for pressure-overload-induced myocardial dysfunction are yet to be fully determined, abnormalities in myocardial intracellular Ca^{2+} handling have been observed in many experimental animal models of pressure-overload hypertrophy and in patients with heart failure (5–7).

The first purpose of this study is to examine whether the age of animals affects mRNA expression of Ca^{2+} -handling proteins in response to pressure overload. To investigate possible mechanisms of myocardial dysfunction associated with pres-

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sure-overload hypertrophy, we analyzed mRNA expression of three SR proteins: Ca^{2+} ATPase, Ca^{2+} release channel (ryanodine receptor), and calsequestrin, and two sarcolemmal proteins: DHP-sensitive Ca^{2+} channel and $\text{Na}^+/\text{Ca}^{2+}$ exchanger in the myocardium subjected to pressure-overload hypertrophy. We studied young (3 mo old) lambs and adult (2 y old) sheep under the identical protocol in which we have extensively characterized systolic and diastolic functions (3, 4).

The second purpose of this study is to determine whether alterations in expressions of Ca^{2+} -handling protein genes occur before the development of pressure-overload hypertrophy. Altered expression of Ca^{2+} regulatory protein genes has been reported in hypertrophied myocardium with clinically overt heart failure (8, 9). However, it is not known whether altered expressions of these genes are pathogenic or are the result of advanced cellular hypertrophy associated with end-stage congestive heart failure. Thus, we also analyzed mRNA levels of Ca^{2+} -handling proteins at 48 h of pressure overload, a time point before the occurrence of hypertrophy or myocardial dysfunction (10).

Therefore, eight groups of animals based on a 3-factorial design (presence or absence of pressure overload $\times$ duration of pressure overload $\times$ age of animals) were studied (Table 1). SR Ca^{2+} ATPase, which revealed a marked decrease in mRNA level only in adult sheep with pressure-overload hypertrophy, was also assessed in terms of both its protein and functional levels, that is, SR Ca^{2+} uptake activity.

This study is unique in that multiple mRNA was evaluated with a parallel hemodynamic assessment in a large animal model.

METHODS

Pressure-overload hypertrophy model. We studied a total of 59 animals in two age groups (3-mo-old lambs and 2-y-old adult Suffolk and Hampshire sheep) with short-term (48 h) and long-term (6 wk) pressure overload induced by ascending aortic banding as well as their age-matched sham-operated controls (Table 1). A subgroup of eight adult sheep with 6 wk of pressure-overload hypertrophy underwent hemodynamic studies as well as mRNA analyses to assess correlation between the mechanical function and mRNA expression of Ca^{2+} -handling proteins. In previous works using the same 6-wk pressure-overload hypertrophy model, we demonstrated depressed systolic and diastolic functions in 2-y-old adult sheep with pressure-overload hypertrophy but normal systolic and

diastolic functions in 3-mo-old lambs with pressure-overload hypertrophy.

The surgical procedure of ascending aortic banding was previously described in detail (3). General anesthesia was induced with 10 mg/kg of intramuscular ketamine and maintained with low-dose halothane and i.v. ketamine ($10 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$). LV and arterial pressure were monitored with a pigtail catheter in the LV through a femoral sheath. A thoracotomy was performed through the third intercostal space. Umbilical tape (U10, Ethicon, Johnson and Johnson, Somerville, NJ, U.S.A.) was wrapped twice around the ascending aorta distal to the coronary arteries and tightened until the peak LV pressure was 30 mm Hg greater than the peak femoral arterial pressure. Surgery in the sham-operated animals was identical including encircling of the aorta with a tape, which was then removed. Penicillin and streptomycin were administered by intramuscular injection before surgery and continued after surgery. Ten adult sheep and six young lambs with the aortic banding died postoperatively, and they were excluded from the study. All of the postoperative deaths occurred during the first 48 h of the aortic-banding surgery except an adult sheep that died 5 wk after the aortic-banding surgery. All the animals used in this study were maintained in accordance with the guidelines of the Children's Hospital Animal Use Committee and the Guide for the Care and Use of Laboratory Animals [DHEW Publication No. 85-23 (National Institutes of Health)].

Extraction of total cellular RNA. The animals were killed 48 h or 6 wk after the surgical procedure with an overdose of pentobarbital (100 mg/kg) and KCl. Total cellular RNA was extracted from the LV tissue by the acid guanidinium thiocyanate-phenol-chloroform method (11). RNA concentration was determined by spectrophotometry with an absorbance at 260 nm.

Northern blot analysis. Samples (20 μg) of total cellular RNA were size fractionated by electrophoresis on a 1.0% agarose/formaldehyde gel and transferred to a nitrocellulose filter. The blots were prehybridized and hybridized as previously described (9, 11) with the following cDNA probes: 1) a 0.7-kb PstI fragment generated from the carboxyl terminal and 3' untranslated region of the cDNA clone specific for the rabbit cardiac/slow twitch skeletal muscle SR Ca^{2+} ATPase (12), 2) a 1.9-kb EcoRI fragment from the cDNA clone IC3A containing the entire coding region and 3' untranslated region of the canine cardiac calsequestrin (13), 3) the 1.8-kb 3' end fragment of human cardiac cDNA encoding $\text{Na}^+/\text{Ca}^{2+}$ exchanger (14)

Table 1. Pressure-overload LV hypertrophy

	48 h		6 wk	
	Aortic banding	Sham operation	Aortic banding	Sham operation
Young lambs:	YB48H	YS48H	YB6W	YS6W
ΔP (mm Hg)	35 ± 25	—	54 ± 6	—
LV/BW (g/kg)	3.04 ± 0.17	3.07 ± 0.07	$4.29 \pm 0.18^{**}$	3.15 ± 0.24
Adult sheep:	AB48H	AS48H	AB6W	AS6W
ΔP (mm Hg)	45 ± 6	—	58 ± 8	—
LV/BW (g/kg)	2.43 ± 0.17	2.46 ± 0.08	$3.52 \pm 0.10^{**}$	2.54 ± 0.12

ΔP , LV-to-aortic peak-to-peak pressure gradient (mm Hg) measured right before death of the animals; LV/BW, LV weight (g)/body weight (kg).

$^{**} p < 0.01$ vs the age-matched controls.

and cDNA inserts of pMHC25, which hybridizes to both α - and β -myosin heavy-chain mRNA (15).

The cDNA inserts were labeled with ^{32}P -dCTP (3000 mCi/mmol, NEN) by the random priming method. After hybridization, the filters were washed serially with the final wash in 0.2X sodium citrate/sodium chloride buffer (SSC buffer) and 0.1% SDS at 55°C for 30 min. The filters were exposed at -80°C on x-ray films. Several exposures were obtained to quantitate relative amounts of each mRNA by laser densitometry in the linear response range of the x-ray films. As a reference point, the mean value of the scores of mRNA from sham-operated adult sheep with 6-wk protocol was arbitrarily set as 1.0.

Slot blot analysis. Samples (5, 10, and 20 μg) of total RNA from individual hearts were loaded onto a nitrocellulose filter using a slot blot apparatus (Schleicher & Schuell, San Diego, CA, U.S.A.). The filter was hybridized with ^{32}P -labeled cDNA probes for the α_1 -subunit of the cardiac DHP-sensitive Ca^{2+} channel, which was a 1.3-kb *EcoRI* fragment isolated from rat cardiac cDNA library (9) and the rabbit cardiac Ca^{2+} release channel (8). Hybridization and washing conditions were the same as those for Northern blot analysis. Several autoradiograms with different exposure time were obtained. The one with the best contrast among the three sample doses (5, 10, and 20 μg) was selected, and the quantification was performed by densitometer scanning.

Isolation of SR vesicles. Isolation of SR vesicles was performed based on a previously described method (16). All the following procedures were done at 4°C. The LV tissue was cut into small pieces and homogenized in 3 mL/g of ice-cold buffer A (50 mM Tris-maleate, pH 6.9, 5 mM NaN_3) using physcotron homogenizer (Nichion, Japan). The homogenate was centrifuged at $1000 \times g$ for 20 min. One-quarter volume of buffer B (50 mM Tris-maleate, pH 6.8, 3 M KCl, 5 mM NaN_3) was added to the supernatant and centrifuged at $14,000 \times g$ for 20 min. The supernatant was centrifuged again at $100,000 \times g$ for 45 min. The pellet was suspended in 3 mL/g of buffer C (20 mM Tris-maleate, pH 6.8, 50 mM KCl), then centrifuged at $100,000 \times g$ for 45 min. The pellet was resuspended in 2 vol of buffer D (30 mM Tris-maleate, pH 6.8, 0.25 M sucrose).

Ca^{2+} uptake activity of SR vesicles. One hundred microliters of the SR sample was preincubated at 30°C for 3 min with 250 μL of buffer E (20 mM Tris-maleate, pH 6.8, 129 mM KCl, 6.43 mM MgCl_2 , 6.43 mM NaN_3 , 0.257 mM EGTA) with 20 mM potassium oxalate and 5 mM Tris-ATP added immediately before the assay. The Ca^{2+} uptake reaction was initiated by adding 50 μL of buffer F (20 mM Tris-maleate, pH 6.8, 100 mM KCl, 5 mM MgCl_2 , 5 mM NaN_3 , 0.2 mM EGTA) with 1940 μM CaCl_2 (2% $^{45}\text{CaCl}_2$). After 2 min of incubation at 30°C, the reaction mixture was filtrated by Millipore filter. The filter was air-dried, and the radioactivity was counted. The Ca^{2+} uptake of the SR vesicle was quantitated as the ratio of cpm counts trapped by the filter, out of a total ^{45}Ca added to the reaction mixture.

Anti-SR Ca^{2+} -ATPase immunoblotting. Tissue homogenate containing 20 μg of protein was electrophoresed on 10% SDS polyacrylamide gels and transferred to a nitrocellulose membrane (Hybond-ECL, Amersham, Buckinghamshire, UK) using a semidry blotting system (Horizon Blot, ATTO, Tokyo,

Japan). Blots were probed by sequential incubation with mouse anti-rat SR Ca^{2+} ATPase (16) and peroxidase-linked donkey anti-mouse IgG. The ECL light-emitting nonradioactive Western blotting kit (Amersham, Buckinghamshire, UK) was used, and light intensity was quantitated by a short exposure to x-ray film.

Hemodynamic study. Ultrasonic dimension gauges for LV internal diameter and wall thickness and an LV pressure manometer were instrumented in eight adult sheep as previously described (3). At the end of 6 wk of pressure overload, the animals were administered 1 mg/kg of propranolol and 0.1 mg/kg of atropine and kept conscious in a nylon sling during the hemodynamic measurement. After a baseline hemodynamic measurement, a stepwise infusion of methoxamine (2.5, 5, 10, and 20 $\mu\text{g}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) was performed to alter afterload and obtain load-independent indices of myocardial contractility.

We developed preload- and afterload-independent assessments of myocardial contractility, midwall shortening fraction (Sm), and midwall shortening rate (SRm) at a common preload (σ_{ed}) and afterload (σ_{aft}) based on the systolic myocardial stiffness concept (3, 17). The indices were previously demonstrated to be applicable to hypertrophied ventricles (3, 10). To assess LV diastolic function, the time constant, tau, of isovolumic relaxation was obtained.

Statistical analyses. Data are expressed as mean $\pm$ SEM. Effects of age, pressure overload, and its duration on each mRNA expression were assessed by the factorial ANOVA. Age differences in responses to pressure overload were examined as a significant interaction in the 3-factorial ANOVA. Individual intergroup differences were assessed by Fischer's exact test if overall significance was demonstrated by ANOVA. Significance was accepted at the level $p < 0.05$.

RESULTS

Pressure-overload LV hypertrophy. The severity of pressure overload (LV-to-aortic pressure gradient) measured immediately before death of the animals did not differ significantly between the two age groups either at 48 h or 6 wk (Table 1). The pressure gradient was greater in 6-wk aortic-banding groups compared with that in 48-h aortic-banding groups in both young and adult animals. This may be due to increased force generation by the hypertrophied myocardium at 6 wk of aortic banding. Aortic banding caused a similar LV hypertrophic response at 6 wk in both age groups (36 and 39% increases in LV-to-body weight ratio in the young and the adult, respectively). No significant LV hypertrophy was observed at 48 h after pressure overload in either age group, indicating that the time point, 48 h, we chose was appropriate to study the early hypertrophic signals before the resultant accumulation of myocardial mass.

Hemodynamic characteristics of the model. We have previously described hemodynamic characteristics of the conscious sheep pressure-overload hypertrophy model used in this study (3, 4). Pressure-overload hypertrophy decreased LV systolic function in the adult sheep. In the current study, 6 wk of pressure overload in adult sheep decreased midwall short-

ening fraction and shortening rate at common preload (50 g/cm^2) and afterload (250 g/cm^2) (Table 2). LV end-diastolic pressure was slightly elevated. Time constant τ at baseline sinus rhythm was $29 \pm 3 \text{ ms}$. Diastolic function reserve, assessed by the time constant of isovolumic LV relaxation during rapid pacing at 270/min, deteriorated in the adult sheep with pressure-overload hypertrophy from $23.5 \pm 0.7 \text{ ms}$ at 120/min to $41.3 \pm 3.9 \text{ ms}$. LV dP/dt max and dP/dt min , which are load-dependent indices of systolic and diastolic function, did not differ between those with and without hypertrophy, as previously shown (3, 4).

Gene expression of Ca^{2+} -handling proteins. Figure 1 shows a representative RNA blot analysis in the adult samples. Each cDNA probe hybridized to single mRNA species of the expected size. Densitometric analysis of multiple samples is shown in Figure 2. The level of the SR Ca^{2+} -ATPase mRNA remained unchanged by aortic banding in the young lambs. In contrast, it decreased by 49 ($p < 0.05$) and 68% ($p < 0.01$) in the adult sheep with aortic banding for 48 h and 6 wk, respectively (Fig. 2A). Three-factorial ANOVA showed a significant interaction between age and pressure overload. Maturation alone did not alter mRNA expression of the SR Ca^{2+} -ATPase gene, because there was no significant difference in the level of this mRNA between the sham-operated young and the adult. Figure 2B shows expression of $\text{Na}^+/\text{Ca}^{2+}$ exchanger mRNA. Adult sheep with 6 wk of aortic banding had 62% less $\text{Na}^+/\text{Ca}^{2+}$ exchanger mRNA than age-matched shams ($p < 0.05$). Three-factorial ANOVA showed significant interaction between age and pressure overload.

Because mRNA levels of the Ca^{2+} release channel of SR and the Ca^{2+} channel are low, we used slot blot analysis. Figure 3 shows statistical results from densitometric analysis. Three-factorial ANOVA showed that pressure overload, regardless of age or duration of pressure overload, slightly increased the mRNA level of the DHP-sensitive Ca^{2+} channel ($p < 0.05$). However, paired comparisons within any of the age and duration groups revealed no significant changes in Ca^{2+} channel mRNA level by the pressure overload.

In contrast, differences in the levels of cardiac calsequestrin, Ca^{2+} release channel, and myosin heavy-chain mRNA among groups did not reach statistical significance (Table 3).

To rule out a potential variation in myocyte/nonmyocyte RNA ratios among groups, we normalized mRNA expression of each of the five genes studied (Ca^{2+} ATPase, $\text{Na}^+/\text{Ca}^{2+}$ exchanger, Ca^{2+} channel, calsequestrin, and

Adult Sheep

Figure 1. Results of representative RNA blot analyses using specific cDNA probes. Bands corresponding to $\text{Na}^+/\text{Ca}^{2+}$ exchanger (7 kb), myosin heavy chain (6.5 kb), SR Ca^{2+} ATPase (4 kb), and calsequestrin (2.9 kb) messages are shown. Each probe hybridized to single mRNA species. Ethidium bromide staining of the gel is shown below.

Ca^{2+} release channel) by the myosin heavy-chain mRNA level of the same sample. The normalized mRNA data of Ca^{2+} -handling proteins revealed the identical results, that is, a decrease in the mRNA level of Ca^{2+} ATPase and $\text{Na}^+/\text{Ca}^{2+}$ exchanger in response to pressure-overload hypertrophy in adult sheep and an increase in DHP-sensitive Ca^{2+} channel mRNA regardless of age.

Ca^{2+} uptake activity of the isolated cardiac SR vesicles. Ca^{2+} uptake activity of the cardiac SR vesicles was depressed only in the adult sheep with the 6 wk of pressure-overload hypertrophy (Table 4), whereas that in the young lambs with the 6 wk of pressure-overload hypertrophy tended to be lower than the age-matched sham control, but the difference did not reach statistical significance.

Table 2. Hemodynamic data of the adult sheep with 6 wk of pressure-overload hypertrophy

	AB6W	Adult sham
LV peak systolic pressure	$165 \pm 12 \text{ mm Hg}^*$	$121 \pm 14 \text{ mm Hg}$
LV end-diastolic pressure	$14 \pm 2 \text{ mm Hg}^*$	$8 \pm 3 \text{ mm Hg}$
Peak aortic pressure	$108 \pm 4 \text{ mm Hg}$	$121 \pm 16 \text{ mm Hg}$
LV-AO pressure gradient	$58 \pm 12 \text{ mm Hg}$	
LV dp/dt max	$2456 \pm 307 \text{ mm Hg/s}$	$2593 \pm 143 \text{ mm Hg/s}$
LV dp/dt min	$2260 \pm 228 \text{ mm Hg/s}$	$2332 \pm 197 \text{ mm Hg/s}$
Tau	$29.3 \pm 3.4 \text{ ms}^*$	$23.8 \pm 1.3 \text{ ms}$
Midwall shortening fraction (preload = 50 g/cm^2 , afterload = 250 g/cm^2)	$12.2 \pm 1.7\%^*$	$20.2 \pm 2.1\%$
Midwall shortening rate (preload = 50 g/cm^2 , afterload = 250 g/cm^2)	$0.39 \pm 0.05^*$	0.64 ± 0.05

* $p < 0.05$ vs adult sham.

Figure 2. (A) Level of the SR Ca^{2+} -ATPase mRNA. The level of SR Ca^{2+} -ATPase mRNA was compared among groups. The mean value of the densitometric scores of mRNA from sham-operated adult sheep on 6-wk protocol was arbitrarily set as 1.0 as a reference point. (B) Level of sarcolemmal $\text{Na}^+/\text{Ca}^{2+}$ exchanger mRNA. The levels of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger mRNA were compared among groups.

Ca^{2+} -ATPase protein levels. Western blotting analysis revealed significantly ($p < 0.01$) decreased SR Ca^{2+} -ATPase protein level (56% of age-matched sham-operated sheep) in adult sheep with the 6 wk of pressure-overload hypertrophy (Fig. 4 and Table 4). In contrast, SR Ca^{2+} -ATPase protein was maintained at a normal level in the young lambs with the same 6 wk of pressure-overload hypertrophy.

Correlations between hemodynamic parameters and mRNA expression of Ca^{2+} -handling proteins. Average values of mRNA expression of Ca^{2+} ATPase and $\text{Na}^+/\text{Ca}^{2+}$ exchanger in a subgroup of eight sheep with 6 wk of pressure overload that underwent hemodynamic study were not different from the average of the entire 13 sheep in the 6-wk pressure-overload group (Fig. 5). There were interindividual variations both in hemodynamic parameters and in mRNA expression. One-to-one correlation was made between each hemodynamic parameter (midwall shortening fraction, shortening rate at

Figure 3. Levels of Ca^{2+} channel mRNA. The levels of the DHP-sensitive Ca^{2+} channel mRNA, determined by slot blot analysis, were higher in groups with LVH (3-factorial ANOVA). The mean value of the scores of mRNA from sham-operated adult sheep with 6-wk protocol was arbitrarily set as 1.0 as a reference point.

common preload and afterload, and tau) and mRNA expression of Ca^{2+} ATPase and $\text{Na}^+/\text{Ca}^{2+}$ exchanger. There were significant correlations between the Ca^{2+} -ATPase mRNA level and midwall shortening fraction ($r = 0.74$, $p < 0.05$), shortening rate ($r = 0.73$, $p < 0.05$), and tau ($r = -0.81$, $p < 0.05$). No significant correlation was detected between $\text{Na}^+/\text{Ca}^{2+}$ exchanger mRNA level and three hemodynamic parameters. Significant correlations were obtained between Ca^{2+} -ATPase protein level and midwall shortening fraction ($r = 0.70$, $p < 0.05$) and shortening rate ($r = 0.71$, $p < 0.05$).

DISCUSSION

We demonstrated decreased mRNA levels of SR Ca^{2+} ATPase and $\text{Na}^+/\text{Ca}^{2+}$ exchanger in adult sheep with pressure-overload hypertrophy. In contrast, in the young lambs, mRNA levels of these myocardial Ca^{2+} -handling proteins were normal even with pressure-overload hypertrophy. The protein level of SR Ca^{2+} ATPase and Ca^{2+} uptake activity of isolated SR vesicles were also depressed only in the adult sheep with pressure-overload hypertrophy, not in the young lambs with pressure-overload hypertrophy. These patterns of biochemical findings were parallel to our previous hemodynamic findings that myocardial systolic and diastolic functions were depressed in the 2-y-old adult sheep but not in the 3-mo-old young lambs with pressure-overload hypertrophy, which supported the hypothesis that abnormalities in myocardial intracellular Ca^{2+} handling play a key role in pressure-overload-induced myocardial dysfunctions. We note that the average value of Ca^{2+} uptake activity in young lambs with pressure-overload hypertrophy tended to be less, although it was statistically nonsignificant. A subclinical level depression of SR function without overt hemodynamic abnormality may have taken place in some young lambs with pressure-overload hypertrophy. Future study with a larger numbers of animals and a broader range of Ca^{2+} correlation in the assay as well as the hemodynamic assessment may give further information.

Table 3. mRNA expression of genes encoding calsequestrin, calcium release channel, and myosin heavy chain

Sham Operation	48 h		6 wk	
	Aortic banding YB48H	Sham operation YS48H	Aortic banding YB6W	Sham operation YS6W
Young lambs:				
Calsequestrin	0.90 $\pm$ 0.20	1.00 $\pm$ 0.36	1.09 $\pm$ 0.22	0.90 $\pm$ 0.15
CRC	0.79 $\pm$ 0.13	0.77 $\pm$ 0.16	1.02 $\pm$ 0.15	1.06 $\pm$ 0.50
MHC	0.97 $\pm$ 0.06	1.11 $\pm$ 0.26	1.07 $\pm$ 0.17	0.99 $\pm$ 0.19
Adult sheep:				
Calsequestrin	0.90 $\pm$ 0.16	0.87 $\pm$ 0.18	0.98 $\pm$ 0.11	1.00 $\pm$ 0.12
CRC	1.06 $\pm$ 0.19	0.96 $\pm$ 0.20	1.15 $\pm$ 0.12	1.00 $\pm$ 0.15
MHC	1.00 $\pm$ 0.19	1.07 $\pm$ 0.10	0.91 $\pm$ 0.13	1.00 $\pm$ 0.11

CRC, calcium release channel; MHC, myosin heavy chain. The mean value of the scores of mRNA from sham-operated adult sheep with 6-wk protocol was arbitrarily determined as 1.0.

Relaxation of the myocardium results from a decrease in cytoplasmic-free Ca^{2+} levels, which is mediated largely by SR Ca^{2+} ATPase. Some animals in the adult pressure-overload hypertrophy (6 wk) group had prolonged tau, and there was a significant inverse correlation between tau and SR Ca^{2+} -ATPase mRNA. Less Ca^{2+} uptake to SR in the diastole results in less Ca^{2+} release from SR in the following systole (16). Thus, depressed systolic function and a positive correlation with the levels of SR Ca^{2+} -ATPase mRNA in this model indicate that the depressed systolic function may also in part be attributable to the decreased SR Ca^{2+} -ATPase expression.

We demonstrated decreases in the $\text{Na}^+/\text{Ca}^{2+}$ exchanger mRNA in the myocardium with pressure-overload hypertrophy in adult sheep. However, no correlation was observed between the hemodynamic parameters and $\text{Na}^+/\text{Ca}^{2+}$ exchanger mRNA levels.

We observed a slightly elevated expression of the α_1 -subunit of the DHP-sensitive Ca^{2+} channel mRNA in pressure overload. Pressure-overload hypertrophy is associated with a prolongation of action-potential duration and decreased rate of repolarization (18), delayed Ca^{2+} transient (19), and prolonged contraction duration (20), which could be explained by a decrease in $\text{Na}^+/\text{Ca}^{2+}$ exchange and an increase in the Ca^{2+} channel activity, although other explanations are possible. We must note that we analyzed mRNA levels of only the α_1 -subunit of the DHP receptor, which is comprised of five subunits. There is a possibility that not all of the subunits are regulated similarly in pressure-overload hypertrophy.

Differences in the levels of expression of calsequestrin and Ca^{2+} release channel mRNA among the groups did not reach statistical significance. This indicates that a decrease in Ca^{2+} ATPase in adult animals is not due to a general decrease in all SR proteins. It is possible that proliferation of fibroblasts is triggered more readily by pressure overload in adult sheep than

in young lambs. Dilution of myocyte RNA with nonmyocyte RNA may result in an underestimation of certain mRNA species (Ca^{2+} ATPase, etc.) and mask overload-induced increases in other species. However, it is unlikely that this is the sole cause of the age-related differences in cardiac gene expression observed in the current study, because the decreases in the Ca^{2+} -ATPase mRNA and $\text{Na}^+/\text{Ca}^{2+}$ exchanger mRNA in the adult with LV hypertrophy (LVH) were evident even when the values were corrected for the level of the myosin heavy-chain mRNA, a myocyte-specific gene product. Normalization of the Ca^{2+} -ATPase mRNA and $\text{Na}^+/\text{Ca}^{2+}$ exchanger mRNA levels by the general increases in protein or DNA level would also deny the possibility of the nonspecific dilution.

Depression of mRNA levels of SR Ca^{2+} ATPase was detected as early as 48 h of pressure overload in adult sheep. This precedes development of hypertrophy (Table 1) and myocardial dysfunction. This suggests that alterations in mRNA expression of Ca^{2+} -handling proteins may be a primary response to pressure overload rather than a late phenomenon secondary to established hypertrophy or heart failure. Therefore, a Ca^{2+} -handling abnormality could be one of the causes of myocardial dysfunction in pressure-overload hypertrophy. There may be some other early hypertrophic signaling that is already inactivated or that is not yet activated at the 48 h of pressure overload. Broader range of the time course would be helpful to elucidate early hypertrophy responses more extensively.

As immature groups, we used 3-mo-old lambs as we did in our previous hemodynamic studies (3, 4). They are large enough for the open-chest surgeries and still under the maturation process. As adult groups, we used 2-y-old Suffolk sheep. Although they are fully matured in terms of body size and reproduction, they corresponded to young adults, considering their longevity of more than 10 y. Thus, an age-related decrease in SR Ca^{2+} -ATPase expression (21) was not likely to

Table 4. Cardiac SR Ca^{2+} ATP protein levels and SR

	YB6W	YS6W	AB6W	AS6W4
Ca^{2+} uptake (nmol/mg/min)	137 $\pm$ 33 (n = 5)	205 $\pm$ 22 (n = 8)	70 $\pm$ 18* (n = 10)	225 $\pm$ 57 (n = 9)
Protein level (relative values)	0.83 $\pm$ 0.11 (n = 5)	0.80 $\pm$ 0.05 (n = 6)	0.56 $\pm$ 0.08** (n = 5)	1.00 $\pm$ 0.14 (n = 5)

The mean value of the protein level scores from sham-operated adult sheep was arbitrarily determined as 1.0. * $p < 0.05$ and ** $p < 0.01$ vs the age-matched controls.

Figure 4. Anti-SR Ca^{2+} -ATPase Western blot. Western blot analysis of the LV tissue homogenate using anti-SR Ca^{2+} ATPase showed a specific binding at approximately 100 kD.

Figure 5. Correlations between Ca^{2+} -ATPase mRNA level and myocardial systolic and diastolic function parameters in adult sheep with pressure-overload hypertrophy. *Shortening fraction* (%), LV midwall fiber shortening fraction at common preload (50 g/cm²) and afterload (250 g/cm²); *shortening rate*, left ventricular midwall fiber shortening rate at common preload (50 g/cm²) and afterload (250 g/cm²); *tau* (ms), time constant of LV isovolumic relaxation. Ca^{2+} -ATPase mRNA level was quantitated as relative values to the mean of sham-operated adult sheep with the 6-wk protocol.

happen in the adult groups of the current study. The young adult sheep at 2 y old may better correspond to young adult patients with congenital or rheumatic disease with pressure-overload hypertrophy and heart failure than to those in their middle age to senescence. In our banded adult sheep, gene expression of $\text{Na}^+/\text{Ca}^{2+}$ exchanger decreased, in contrast with many previous reports of its increase in animal models and patients with hypertrophy and heart failure. Older adult sheep would have different results from those we observed in the 2-y-old young adult sheep. On the other hand, lambs younger than 3-mo-old would have shown developmental changes in Ca^{2+} handling as reported in the literature (22).

Another age-related limitation of the current study comes from the smaller LV size and growth in the lamb groups. A potential criticism is that the lambs were exposed to a gradually induced pressure overload because of their growth (3), whereas the onset of pressure overload was more abrupt in the adult sheep. Initial increases in LV wall stress were set to be

equivalent to the similar increase in LV pressure, assuming that LV geometry was not substantially changed acutely by the banding surgery. The LV-to-aortic pressure gradient was similar between the lambs and adult sheep even at 6 wk of pressure overload. However, the smaller hearts of the banded lambs may adapt to the pressure increase with less increase in wall stress. Doppler flow study would have provided us with more detailed information regarding the actual circumference of the aorta *versus* the area of the band, allowing us to quantify the relative degree of reduced flow.

We examined expression of the cardiac genes only at the mRNA levels except for SR Ca^{2+} ATPase in which both protein level and Ca^{2+} uptake activity were evaluated. It is possible that some translational or posttranslational controls may be present in regulation of these genes. Because the sarcolemmal Ca^{2+} channel and the SR Ca^{2+} release channel were quantitated by slot blot analysis using total RNA because of less abundance of these mRNA species, possible nonspecific bindings of the probes to ribosomal RNA and individual variations in some of the slot blot analysis data might have blunted actual small alterations in mRNA levels. Large SEM in the slot blot analysis data in some groups indicates the methodologic limitation. It may not be appropriate to extrapolate these slot blot-derived mRNA data to protein levels. Indeed, a recent study (23) showed reduced ryanodine receptor (Ca^{2+} release channel) density and no changes in DHP receptor (Ca^{2+} channel) numbers in guinea pigs and ferrets with severe LV pressure-overload hypertrophy. However, other studies have shown that the mRNA and protein levels (7, 24, 25) as well as the Ca^{2+} uptake rate (26) of the SR Ca^{2+} ATPase changed in a concordant manner in LV myocardium subjected to pressure overload and in patients with heart failure. Although yet to be proven, it is likely that the changes in the mRNA levels in the current study reflect changes in levels of the corresponding proteins. Assessment of alterations of intracellular Ca^{2+} -cycling properties using Ca^{2+} indicators would provide us with more direct evidence for the relationship between the Ca^{2+} -handling abnormality and hemodynamic dysfunction in the banded adult sheep. Further studies are necessary to prove this point, especially in the SR Ca^{2+} release channel and the sarcolemmal Ca^{2+} channel. α - and β -myosin heavy chain are differentially regulated in response to pressure overload. Because the cDNA we used hybridizes both subtypes, the subtype shift of myosin heavy chain may have taken place in the banded animals. However, it is widely accepted that β -myosin heavy-chain expression is altered in pressure-overload hypertrophy primarily in small animals and not in large animals. We note that the myosin heavy-chain mRNA data were employed only for the normalization of the Ca^{2+} -handling protein mRNA.

REFERENCES

1. Dowell RT, McManus RE 1978 Pressure-induced cardiac enlargement in neonatal and adult rats: left ventricular functional characteristics and evidence of cardiac muscle cell proliferation in the neonate. *Circ Res* 42:303-310
2. Donner R, Caraballo BA, Black I, Spann JF 1983 Left ventricular wall stress in compensated aortic stenosis in children. *Am J Cardiol* 51:946-951
3. Aoyagi T, Mirsky I, Flanagan MF, Currier JJ, Colan SD, Fujii AM 1992 Myocardial function in immature and mature sheep with pressure-overload hypertrophy. *Am J Physiol* 262:H1036-H1048

4. Fujii AM, Aoyagi T, Flanagan MF, Takahashi T, Bennett-Guerrero E, Colan SD, Izumo S 1993 Response of the hypertrophied left ventricle to tachycardia: importance of maturation. *Am J Physiol* 264:H983-H993
5. Schwartz K, Chassagne C, Boheler KR 1993 The molecular biology of heart failure. *J Am Coll Cardiol* 22(suppl A):30A-33A
6. Morgan JP, Erny RE, Allen PD, Grossman W, Gwathmey JK 1990 Abnormal intracellular calcium handling, a major cause of systolic and diastolic dysfunction in ventricular myocardium from patients with heart failure. *Circulation* 81(suppl):III21-III32
7. Arai M, Matsui H, Periasamy M 1994 Sarcoplasmic reticulum gene expression in cardiac hypertrophy and heart failure. *Circ Res* 74:555-564
8. Brillantes AM, Allen P, Takahashi T, Izumo S, Marks AR 1992 Differences in cardiac calcium release channel (ryanodine receptor) expression in myocardium from patients with end-stage heart failure caused by ischemic *versus* dilated cardiomyopathy. *Circ Res* 71:18-26
9. Takahashi T, Allen PD, Lacro RV, Marks AR, Dennis AR, Schoen FJ, Grossman W, Marsh JD, Izumo S 1992 Expression of dihydropyridine receptor (Ca^{2+} channel) and calsequestrin genes in the myocardium of patients with end-stage heart failure. *J Clin Invest* 90:927-935
10. Aoyagi T, Fujii AM, Flanagan MF, Arnold LW, Colan SD, Brathwaite KW, Mirsky I 1993 Transition from compensated hypertrophy to intrinsic myocardial dysfunction during development of left ventricular pressure-overload hypertrophy in conscious sheep. Systolic dysfunction precedes diastolic dysfunction. *Circulation* 88:2415-2425
11. Takahashi T, Schunkert H, Itoyama S, Wei JY, Nadal-Ginard B, Grossman W, Izumo S 1992 Age-related differences in the expression of proto-oncogene and contractile protein genes in response to pressure overload in the rat myocardium. *J Clin Invest* 89:939-946
12. MacLennan DH, Brandl CJ, Korczak B, Green NM 1985 Amino-acid sequence of a Ca^{2+} - Mg^{2+} -dependent ATPase from rabbit muscle sarcoplasmic reticulum, deduced from its complementary DNA sequence. *Nature* 316:696-700
13. Scott BT, Simmerman HKB, Collins JH, Nadal-Ginard B, Jones LB 1988 Complete amino acid sequence of canine cardiac calsequestrin deduced by cDNA cloning. *J Biol Chem* 263:8958-8964
14. Komuro I, Wenninger KE, Philipson KD, Izumo S 1992 Molecular cloning and characterization of the human cardiac $\text{Na}^{+}/\text{Ca}^{2+}$ exchanger cDNA. *Proc Natl Acad Sci USA* 89:4769-4773
15. Strehler EE, Strehler-Page MA, Perriard JC, Periasamy M, Nadal-Ginard B 1986 Complete nucleotide and encoded amino acid sequence of a mammalian myosin heavy chain gene. Evidence against intron-dependent evolution of the rod. *J Mol Biol* 190:291-317
16. Wimsatt DK, Hohl CM, Brierley GP, Altschuld RA 1990 Calcium accumulation and release by the sarcoplasmic reticulum of digitonin-lysed adult mammalian ventricular cardiomyocytes. *J Biol Chem* 265:14849-14857
17. Aoyagi T, Fujii AM, Colan SD, Flanagan MF, Mirsky I 1992 Different responses of extent and velocity of contraction to dobutamine in conscious sheep. *Am J Physiol* 263:H1250-H1261
18. Nordin C, Siri F, Aronson RS 1989 Electrophysiologic characteristics of single myocytes isolated from hypertrophied guinea pig hearts. *J Mol Cell Cardiol* 21:729-739
19. Gwathmey JH, Morgan JP 1985 Altered calcium handling in experimental pressure-overload hypertrophy in the ferret. *Circ Res* 57:836-843
20. Aoyagi T, Momomura S, Matsui H, Serizawa T, Iizuka M, Hirata Y, Sugimoto T, Mirsky I 1991 Myocardial contractility and contraction duration are unrelated in the left ventricle with moderate pressure-overload hypertrophy. *Jpn Heart J* 32:111-130
21. Lompre AM, Lambert F, Lakatta EG, Schwartz K 1991 Expression of sarcoplasmic reticulum $\text{Ca}(2+)$ -ATPase and calsequestrin genes in rat heart during oncogenic development and aging. *Circ Res* 69:1380-1388
22. Studer R, Reinecke H, Vetter R, Holtz J, Drexler H 1997 Expression and function of the cardiac $\text{Na}^{+}/\text{Ca}^{2+}$ -exchanger in postnatal development of the rat, in experimental-induced cardiac hypertrophy, and in the failing human heart. *Basic Res Cardiol* 92(suppl 1):53-59
23. Rannou F, Sainte-Beuve C, Oliviero P, Do E, Trouve P, Charlemagne D 1995 The effects of compensated cardiac hypertrophy on dihydropyridine and ryanodine receptors in rat, ferret, and guinea pig hearts. *J Mol Cell Cardiol* 27:1225-1234
24. De la Bastie D, Levitsky D, Rappaport L, Mercadier JJ, Marotte F, Wisniewsky C, Brovkovich V, Schwartz K, Lompre AM 1990 Function of the sarcoplasmic reticulum and expression of its Ca^{2+} -ATPase gene in pressure overload-induced cardiac hypertrophy in the rat. *Circ Res* 66:554-564
25. Movsesian MA, Karimi M, Green K, Jones LR 1994 Ca^{2+} -transporting ATPase, phospholamban, and calsequestrin levels in nonfailing and failing human myocardium. *Circulation* 90:653-657
26. Kiss E, Ball NA, Kranias EG, Walsh RA 1995 Differential changes in cardiac phospholamban and sarcoplasmic reticular Ca^{2+} -ATPase protein levels. Effects on Ca^{2+} transport and mechanics in compensated pressure-overload hypertrophy and congestive heart failure. *Circ Res* 77:759-764