Original Article

High Ambient Pressure Produces Hypertrophy and Up-Regulates Cardiac Sarcoplasmic Reticulum Ca^{2+} Regulatory Proteins in Cultured Rat Cardiomyocytes

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Previously, we demonstrated *in vivo* that the nature of the alterations in sarcoplasmic reticulum (SR) function and SR Ca^{2+} regulatory proteins depends both on the type of mechanical overload imposed and on the duration of the heart disorder. The purpose of the present study was to determine *in vitro* whether an extrinsic mechanical overload (in the form of high ambient pressure) would cause an up-regulation of ryanodine receptor (RyR) and Ca^{2+}-ATPase, as we previously reported mildly pressure-overloaded, hypertrophied rat hearts. Primary cultures of neonatal rat cardiomyocytes were prepared and high ambient pressure was produced using an incubator and pressure-overloading apparatus. Cells were exposed to one of two conditions for 72 h: atmospheric pressure conditions (APC) or high pressure conditions (HPC; HPC = APC + 200 mmHg). The expression levels of RyR and Ca^{2+}-ATPase were quantified and functional characteristics were monitored. The cell area was significantly greater under HPC. After 6 h exposure, the physiological properties of cardiomyocytes were impaired, but they returned to the baseline level within 24 h. After 24 h exposure, the expression level of RyR was significantly higher under HPC, and for Ca^{2+}-ATPase, the expression level was significantly higher under HPC after 6 h exposure. HPC caused hypertrophy and up-regulated the expression of Ca^{2+} regulatory proteins and their genes. We suggest that this *in vitro* pressure-overloading model may prove useful, as is a stretch-overloading model, for investigation of the intracellular Ca^{2+} regulatory pathways responsible for the development of cardiac hypertrophy. (*Hypertens Res* 2006; 29: 1013–1020)

***Key Words*: cardiomyocyte, pressure-overload, hypertrophy, ryanodine receptor, Ca^{2+}-ATPase**

Introduction

The cardiac sarcoplasmic reticulum (SR) plays an important role in excitation-contraction (E-C) coupling and, consequently, abnormalities of its Ca^{2+} release and uptake functions may result in systolic and diastolic dysfunctions, as in cardiac hypertrophy and heart failure. The contraction of cardiomyocytes is triggered by Ca^{2+} release from the SR, *via* a Ca^{2+} release channel that is also referred to as the ryanodine receptor (RyR). Their subsequent relaxation is initiated by an ATP-dependent transport of Ca^{2+} (*via* Ca^{2+}-ATPase) back into SR. Alterations in Ca^{2+} transport by the SR have been reported to be a cause of the altered cardiac function seen in cardiac hypertrophy and heart failure (*1*, *2*). A number of groups have identified a decrease in the level of RyR and an SR Ca^{2+}-

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Table 1. Effects of Pressure-Overload on Cell Area (μm^2)

	APC	HPC
0 h	565±78	
6 h	564±93	835±154*,#
12 h	678±134#	931±162*,#
24 h	726±157#	993±245*,#
48 h	602±137	830±211*,#
72 h	633±144#	769±188*,#

APC, atmospheric pressure conditions; HPC, high pressure conditions. *$p<0.01$ *vs*. APC, #$p<0.05$ *vs*. 0 h. Mean±SD.

ATPase down-regulation in both human and experimental heart failure *in vivo* (*1*). Previously, we have reported alterations in cardiac function, in SR Ca^{2+} release and uptake functions, and in the expression of SR Ca^{2+} regulatory proteins (RyR and/or Ca^{2+}-ATPase) during the development of cardiac hypertrophy and heart failure in *in vivo* animal models (*3*–*6*). Our studies revealed that the nature of the alterations in SR function and SR Ca^{2+} regulatory proteins depended on the type of mechanical overload imposed and on the different duration of the heart disorder in these models. For instance, volume-overloaded hypertrophic rat hearts exhibited reduced SR functions and a decreased number of RyR, even in the hemodynamically compensatory (adaptive) stage (*3*). In contrast, in mildly pressure-overloaded rat hearts, cardiac hypertrophy was associated with an enhancement of the Ca^{2+} uptake and release functions of the SR and an up-regulated number of RyR during the early stage of the development of the hypertrophy (*4*). Interesting though these descriptive studies were, they did not identify the intracellular mechanisms responsible for the alterations in SR function and SR Ca^{2+} regulatory proteins induced by mechanical overload. Cultured neonatal rat cardiomyocytes have proven to be useful tools in our struggle to understand the cellular mechanisms involved in the regulation of SR Ca^{2+} regulatory proteins that occurs in response to mechanical factors. Although we have demonstrated that different types of cardiac-hypertrophy–inducing mechanical overload have different effects on SR Ca^{2+} regulatory proteins in *in vivo* animal models, it is currently unknown whether similar alterations are produced in response to extrinsic mechanical overload. Recently, Cadre *et al*. (*7*) reported that cyclic stretch caused a down-regulation of Ca^{2+}-transporter–gene expression in neonatal rat ventricular myocytes. Their results suggested that an *in vitro* model system should prove useful as a means of dissecting the cellular response during cardiac hypertrophy and heart failure *in vivo*.

The purpose of the present study was to determine whether, *in vitro*, an extrinsic mechanical overload (in the form of a high ambient pressure) would cause hypertrophy and an up-regulation of SR Ca^{2+} regulatory proteins (RyR and Ca^{2+}-ATPase) of the type previously reported by us in mildly hypertrophied rat hearts *in vivo*.

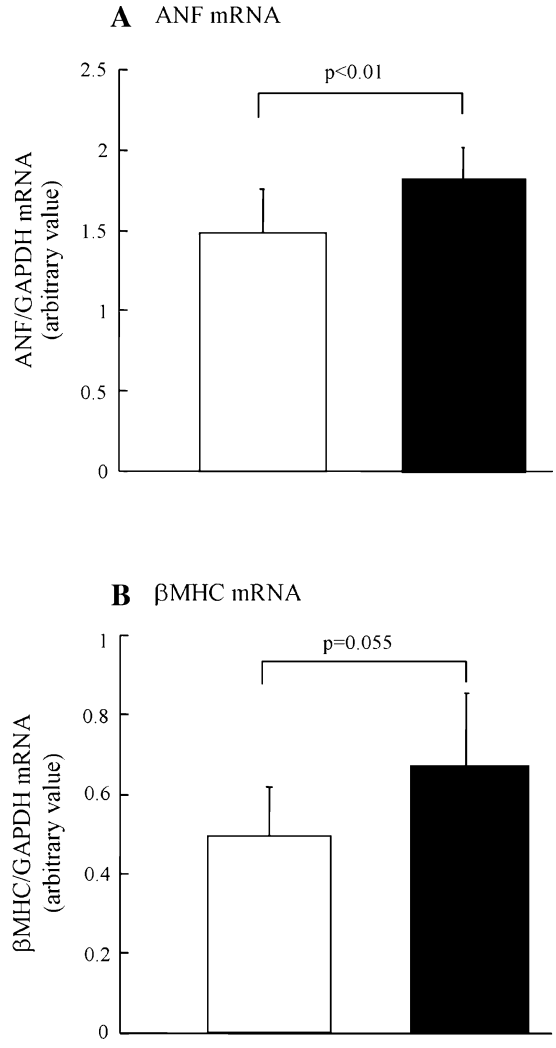

Fig. 1. *Expression levels of the mRNAs encoding atrial natriuretic factor (ANF) (A) and β-myosin heavy chain (βMHC) (B) in the cardiomyocytes either under atmospheric pressure conditions (APC, open bars) or under high pressure conditions (HPC, closed bars) for 24 h. Data are from 6 independent experiments in each group (mean±SD).*

Methods

Cell Culture

The animals used for this study were handled in accordance with the Guiding Principles in the Care and Use of Animals, as approved by the Animal Care Committee of Yamaguchi University Graduate School of Medicine. In addition, this investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Primary cultures of neonatal rat cardiomyocytes were pre-

pared using the method originally described by Simpson *et al*. with minor modifications (*8*). Briefly, hearts from 1- or 2-day-old Wistar rats were minced, then dissociated with the aid of collagenase. After incubation of dispersed cells on 100-mm culture dishes (Falcon, Oxnard, USA) for 60 min at 37°C, non-attached viable cells were collected and seeded into 35-mm dishes (4×10^5 cells per dish). Cardiomyocytes were incubated in Leibovitz L-15 Medium (Worthington Biochemical Co., Lakewood, USA) supplemented with 10% fetal bovine serum and 0.1 mmol/l bromodeoxyuridine (BrDu) for the first 72 h. The serum was then replaced with 0.5% serum medium, and the cardiomyocytes were incubated for an additional 24 h. The cells were placed in the high pressure apparatus for 0–72 h. In certain experiments, we prepared cultures of almost pure monolayer cardiomyocytes with synchronous beating by the use of 0.1 mmol/l BrDu for the first 72 h after plating so as to inhibit fibroblast growth; this preferentially reduces the proportion of nonmyocytes as previously described (*9*). The percentage of cardiomyocytes was >95% at the start of the experiments.

Application of High Ambient Pressure

High ambient pressure was produced using the incubator and pressure-overloading apparatus previously developed in our laboratory (*10*). In brief, a small resealable acrylic chamber was designed to fit inside a standard incubator. This chamber had three openings: entrance and exit ports for compressed air and a hole for passing wires to both a pCO_2/pO_2 sensor (Microgas 7640; Kontron Instruments, Tokyo, Japan) and a pH sensor (Digitrapper Mk III; Synectics Medical, Stockholm, Sweden). A constant pressure chosen from within the range 0 to 1,013.3 hPa (0–760 mmHg) could be achieved by adding compressed air to the chamber to an appropriate extent. By changing the composition of the compressed air in advance, we could keep the pO_2 and pCO_2 concentrations of the air in the small chamber almost equal to those of atmospheric air (pO_2=205±0.7 hPa, pCO_2=49±0.7 hPa). In this study, cells were exposed to two conditions at 37°C: atmospheric pressure conditions (APC) and high pressure conditions (HPC). Measurement confirmed that the pH was identical (pH=7.4±0.05) in each of the mediums used under either of these two conditions. The HPC involved use of a pressure 200 mmHg above normal atmospheric pressure. This values was chosen because the use of a pressure 100 mmHg above normal atmospheric pressure caused no significant changes in neonatal cardiomyocytes and a pressure 300 mmHg above normal atmospheric pressure caused cell weakness (data not shown).

Measurement of Physiological and Morphological Characteristics

Physiological characteristics were monitored with a video-based edge-detection system (Ion Optix Co., Milton, USA) as previously reported (*11*). In brief, individual cardiomyocytes were placed on the stage of an inverted microscope (Eclipse TS100; Nikon, Tokyo, Japan) and displayed on a computer monitor by means of an Ion Optix MyoCam camera. Soft-edge-software (Ion Optix Co.) was then used to analyze the cardiomyocytes for cell length during shortening and relengthening. These physiological characteristics were measured for 72 h in each dish. The physiological performance of cardiomyocytes in 5 random fields (5 cells/field) was measured from 6 independent experiments.

For the measurement of cell size, cardiomyocytes were placed in 0.5% serum medium on a culture slide (BIOCOAT; Becton Dickinson Labware, Bedford, USA), cultured in the high ambient pressure apparatus under one of the pressure conditions for 0–72 h, then fixed with 10% formaldehyde solution in phosphate-buffered saline. Cell area was evaluated in haematoxylin-eosin preparations using a public image analysis application (NIH image). The cell size of cardiomyocytes in 10 random fields (50 cells/field) was measured from 6 independent experiments.

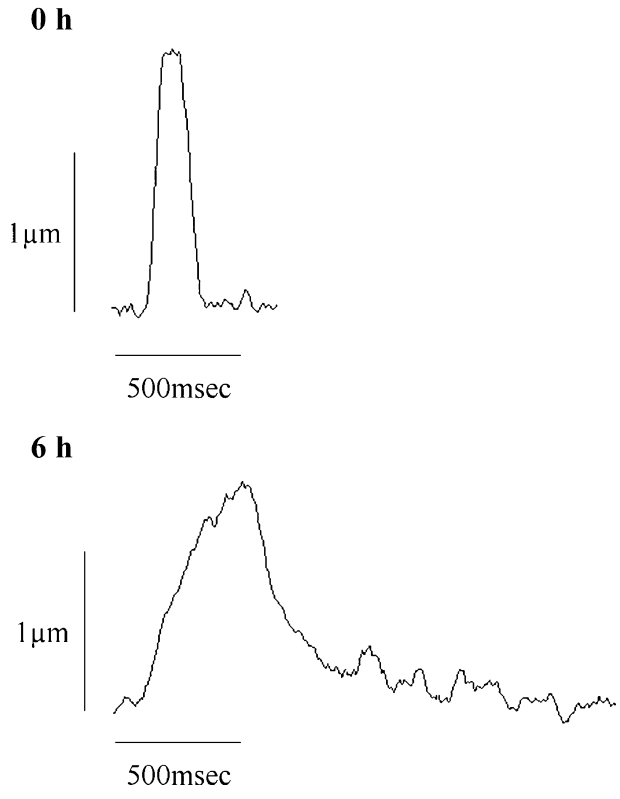

Fig. 2. *Effects of high pressure condition on physiological characteristics of cardiomyocytes. Examples of the cell contraction-relaxation in a cardiomyocyte exposed to high pressure for 0 h or 6 h are shown.*

Table 2. Effects of Pressure-Overload on Physiological Characteristics of Cardiomyocytes

	T_{peak} (ms)	T_{70} (ms)	$+dL/dt$	$-dL/dt$
0 h	107±44	99±28	26.53±2.83	16.38±2.04
6 h	274±128$^{\#\#}$	165±6$^{\#}$	8.94±1.25$^{\#}$	12.29±6.23
24 h	121±61	170±80$^{\#}$	31.28±4.47	14.85±3.05
48 h	91±6	92±30	23.09±3.57	15.59±3.62
72 h	79±4	65±23	25.17±4.75	26.14±3.90$^{\#}$

T_{peak}, time to peak tension; T_{70}, time to 70% tension-regression; $+dL/dt$, maximal rate of shortening; $-dL/dt$, maximal rate of re-lengthening; $^{\#}p<0.05$, $^{\#\#}p<0.01$ *vs*. baseline (0 h). Mean±SD.

Western Blotting Analysis

Cells were lysed in protein lysis buffer (50 mmol/l Tris-HCl, pH 7.5, containing 150 mmol/l NaCl, 0.5% Nonidet P-40, 50 mmol/l NaF, 1 mmol/l Na_3VO_4, 1 mmol/l DTT, 1 mmol/l PMSF, 25 μg/ml leupeptin and 25 μg/ml aprotinin). Protein concentration was determined by a Bio-Rad Dc protein assay (Bio-Rad Laboratories, Hercules, USA). Immunoblot analysis was performed as previously described, with some modifications (*4*, *5*). Equal amounts of protein (80 μg/lane for RyR and 20 μg/lane for Ca^{2+}-ATPase) were electrophoretically separated on SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The RyR and Ca^{2+}-ATPase protein bands were detected using monoclonal anti–ryanodine receptor antibody (Affinity Bioreagents, Inc., Golden, USA) and anti-SERCA2 ATPase antibody (Affinity Bioreagents, Inc.), respectively. The amount of protein recognized by the antibodies was quantified by means of an ECL immunoblotting detection system (Amersham, Bucks, UK), with the membrane being exposed to X-ray film. Quantitative densitometry of immunoblots was performed using a microcomputer imaging device (AE-6900M; ATTO, Tokyo, Japan).

RNA Preparation

Total cellular RNA was isolated from each frozen cell sample by the acid guanidinium thiocyanate/phenol/chloroform extraction method (*12*), then stored at −80°C.

Reverse Transcription and Polymerase Chain Reaction Amplification

The appropriate cDNAs were prepared using a Takara RNA PCR Kit (Takara, Tokyo, Japan), as previously described (*13*). The primers for the amplification of RyR, Ca^{2+}-ATPase, atrial natriuretic factor (ANF), β-myosin heavy chain (βMHC) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed from published sequences. For the RyR, they were based on the following rat sequences (GenBank accession number U95157): at positions 455–483 (sense primer, 5′-GAGTCAGCATTCTGGAAGAAAATC ATAGC-3′) and 1191–1215 (antisense primer, 5′-GACATG GTCATATACCAGGCTAGGT-3′) (predicted length of the polymerase chain reaction [PCR] product, 761 bp). For the Ca^{2+}-ATPase, they were based on rat sequences (*14*) at positions 2951–2974 (sense primer, 5′-TGTCTGAAAACC AGTCCCTGCTGA-3′) and 3175–3199 (antisense primer, 5′-ATGGACCTCGGACGTTATGACCTCA-3′) (predicted length of the PCR product, 249 bp). For ANF, they were based on rat sequences (*15*) at positions 1–24 (sense primer, 5′-ATGGGCTCCTTCTCCATCACCAAG-3′) and 427–459 (antisense primer, 5′-TTATCTTCGGTACCGGAAGCT GTTGCAGCCTAG-3′) (predicted length of the PCR product, 459 bp). For βMHC, they were based on rat sequences (GenBank accession number X15939) at positions 5549–5569 (sense primer, 5′-ATCAAGGAGCTCACCTACCAG-3′) and 5864–5883 (antisense primer, 5′-GTC TGTTTCAAAGGCTCCAG-3′) (predicted length of the PCR product, 335 bp). For GAPDH, they were based on human sequences (*16*) at positions 102–125 (sense primer, 5′-CTTCATTGACCTCAACTACATGGT-3′) and 805–828 (antisense primer, 5′-CTCAGTGTAGCCCAGGATGCC CTT-3′) (predicted length of the PCR product, 726 bp).

Quantitation of PCR Products

The optimal number of amplification cycles needed to allow quantitation of RyR, Ca^{2+}-ATPase, ANF, βMHC and GAPDH gene PCR products was determined. The PCR products for each cycle were subjected to 5% polyacrylamide gel electrophoresis (PAGE) and autoradiography, then the associated radioactivity was measured using an imaging analyzer (model BAS-2000; Fuji Photo Film Co., Tokyo, Japan). The optimal number of cycles was found to be 25 for RyR, Ca^{2+}-ATPase, ANF, βMHC and GAPDH.

Assessment of the Expression of RyR, Ca^{2+}-ATPase, ANF and βMHC mRNAs

The relative radioactivity associated with RyR, Ca^{2+}-ATPase, ANF or βMHC PCR products in each sample was calculated by dividing the radioactivity associated with the RyR, Ca^{2+}-ATPase, ANF or βMHC PCR products by the radioactivity associated with the GAPDH gene product (internal control; amplified simultaneously). Each level of reverse transcription and PCR (RT-PCR) product was obtained from six indepen-

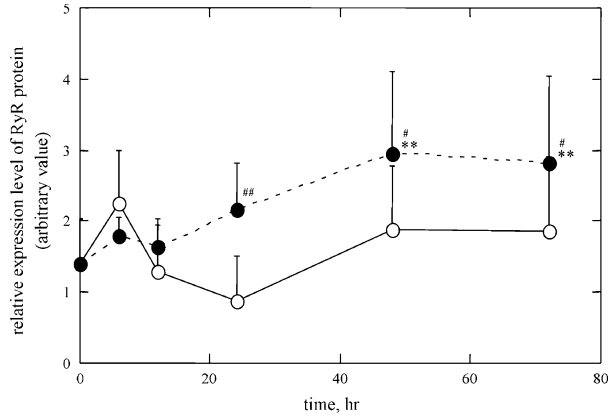

Fig. 3. *Time course of the effect of high ambient pressure on the relative expression level of RyR protein. Open circles, expression level under atmospheric pressure conditions (APC); closed circles, under high pressure conditions (HPC). Data are from 6 independent experiments in each group (mean±SD).* **p < *0.01* vs. *0 h,* #p < *0.05* vs. *APC,* ##p < *0.01* vs. *APC.*

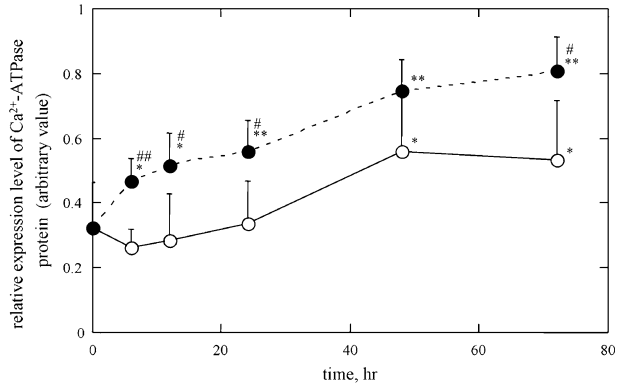

Fig. 4. *Time course of the effect of high ambient pressure on the relative expression level of Ca^{2+}-ATPase protein. Open circles, expression level under atmospheric pressure conditions (APC); closed circles, under high pressure conditions (HPC). Data are from 6 independent experiments in each group (mean±SD).* *p < *0.05* vs. *0 h,* **p < *0.01* vs. *0 h,* #p < *0.05* vs. *APC,* ##p < *0.01* vs. *APC.*

dent experiments in each group. We used GAPDH as an internal control because the densitometric scores for the mRNAs did not differ between the groups of cells. Furthermore, this enzyme of the glycolytic pathway is constitutively expressed in most tissues and is the most widely accepted internal control in the molecular biology literature (*7*, *17*).

Statistical Analysis

All data are presented as the mean±SD. Comparisons between data were made using analysis of variance (ANOVA) followed by Fisher's test and Student's unpaired *t*-test. Differences were taken to be significant at $p<0.05$.

Results

Cardiomyocyte Characteristics

The application of high ambient pressure to neonatal cardiomyocytes produced myocyte hypertrophy. Tables 1 shows that the myocyte area was significantly greater in the HPC group than in the APC group at all timepoints. As seen in Fig. 1, at 24 h, the expression level of ANF mRNA in the HPC group (1.82±0.27) was significantly higher than that in the APC group (1.50±0.27, $p<0.01$). Moreover, ANF secretion in the HPC group (6.2±1.0 ng/ml) was significantly higher than in the APC group (3.8±0.2 ng/ml, $p<0.01$). The expression level of βMHC mRNA in the HPC group (0.67±0.18) was higher than that in the APC group (0.50±0.12), but the difference did not reach the level of statistical significance ($p=0.055$). There was no significant difference in the relative spontaneous beating rate, which was calculated by dividing the beating rate at each timepoint by the beating rate before the exposure (at 0 h), between the APC and HPC groups (data not shown). Both the example (Fig. 2) and the summary data (Table 2) show the physiological characteristics of cardiomyocytes exposed to HPC. After 6 h exposure, HPC caused a transient impairment of contraction-relaxation performances, but the contraction properties of cardiomyocytes returned to the baseline level within 24 h. Interestingly, after 72 h the rate of cell re-lengthening ($-dL/dt$) increased significantly compared with that at baseline. In the APC group, there were no significant differences in the physiological characteristics of cardiomyocytes over the 72-h culture period.

Analysis of the Expression Levels of RyR and Ca^{2+}-ATPase Proteins

The expression level of RyR protein (Fig. 3) in the HPC group gradually increased after the exposure began and, at 24 h, the expression level was significantly greater than in the APC group (2.16±0.67 *vs*. 0.88±0.62, $p<0.05$). Moreover, at 48 h, the expression level under HPC (2.94±1.17) was significantly higher than that at 0 h in the same group (1.41±0.63, $p<0.05$). The up-regulation continued for 72 h under HPC. In contrast, although there was a slight but not significant decrease in the expression of RyR protein at 24 h, there was no significant change in the expression level of RyR protein under APC over the 72 h of the experiment. Interestingly, in the case of Ca^{2+}-ATPase protein (Fig. 4), the expression level gradually increased not only under HPC, but also under APC. Under HPC, the expression level of Ca^{2+}-ATPase protein at 6 h was significantly greater than that under APC at the same timepoint (0.47±0.07 *vs*. 0.26±0.06, $p<0.01$) and significantly greater than at time 0 h (0.33±0.14, $p<0.05$), and the

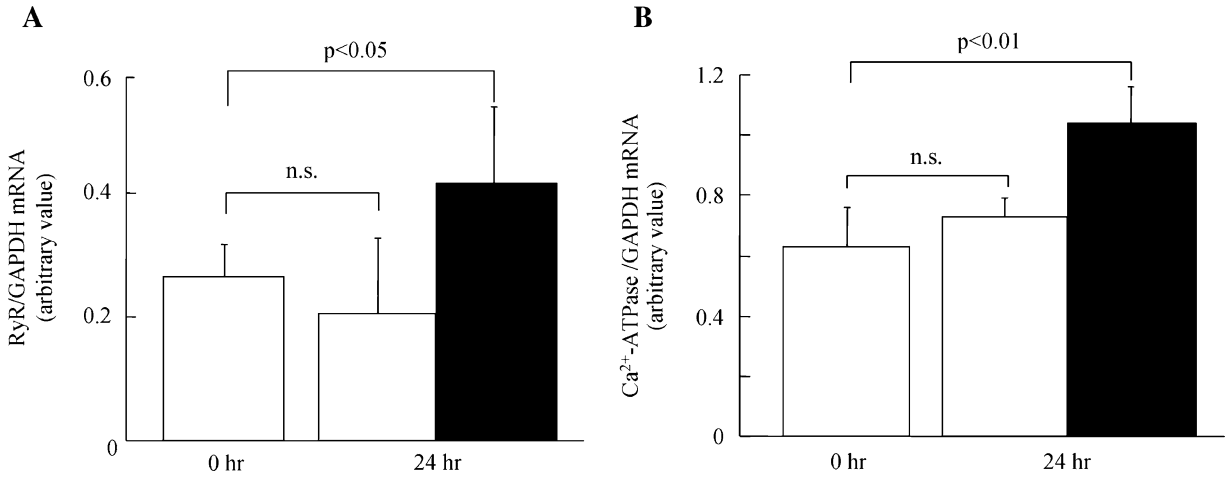

Fig. 5. *Expression levels of the mRNAs encoding RyR (A) and Ca^{2+}-ATPase (B) after high pressure exposure. Neonatal cardiac myocyte cultures were maintained under atmospheric pressure conditions (APC, open bars) or high pressure conditions (HPC, closed bar). Under HPC, the relative expression levels of RyR and Ca^{2+}-ATPase mRNA were significantly increased, but under APC there was no significant difference in the expression levels of either mRNA between 0 h and 24 h. Data are the mean±SD from 6 independent cell isolations.*

up-regulation persisted until 72 h after the start. Under APC, the expression of Ca^{2+}-ATPase protein was significantly greater at 48 h than at 0 h (0.56±0.18 *vs*. 0.33±0.14, $p<0.05$), and the increase persisted until the end of the experiment at 72 h.

Analysis of the Expression Levels of the mRNAs for RyR and Ca^{2+}-ATPase

As indicated in Fig. 5, high ambient pressure significantly increased the relative expression levels of the mRNAs encoding RyR and Ca^{2+}-ATPase. These increased levels persisted until 72 h of exposure (data not shown). Under HPC, the relative expression level of RyR mRNA was significantly increased after 24 h of exposure (from 0.269±0.056 to 0.414±0.123; $p<0.05$) (Fig. 5A) and the relative expression level of Ca^{2+}-ATPase mRNA was also significantly increased after 24 h of exposure (from 0.635±0.129 to 1.030±0.121; $p<0.01$) (Fig. 5B). Under APC, there was no significant difference in the expression levels of either of the mRNAs between 0 h and 24 h (at 24 h: 0.233±0.102 for RyR mRNA and 0.735±0.060 for Ca^{2+}-ATPase mRNA). Interestingly, at 24 h the relative expression level of the Ca^{2+}-ATPase mRNA under APC (0.735±0.060) tended to show an increase, although the difference was not significant.

Discussion

The major findings of the present study were as follows: 1) we showed that an *in vitro* high pressure-overload model system for cultured cardiac myocytes can be used to investigate alterations in SR Ca^{2+} regulatory proteins during the development of hypertrophy; 2) both RyR and Ca^{2+}-ATPase expressions were up-regulated in hypertrophied cardiac myocytes subjected to a high ambient pressure, contributing at least in part to the maintenance of contraction-relaxation characteristics in cardiac myocytes. To our knowledge, although there have been many investigations of the alterations in cardiac SR Ca^{2+} regulatory proteins associated with stretch-induced myocyte hypertrophy *in vitro*, this is the first investigation in which changes in these proteins have been evaluated under high ambient pressure conditions in cultured cardiac myocytes. Due to the complex *in vivo* physiology of cardiac hypertrophy, it is very difficult to define the signaling mechanisms responsible for the alterations in cardiac myocyte gene expression that in turn may contribute to the pathogenesis of this disorder. Cultured neonatal rat myocytes have been a useful model for clarifying the mechanisms involved in the regulation of SR Ca^{2+} regulatory proteins that occurs in response to mechanical factors.

As described in Introduction, recent studies have demonstrated that the alterations occurring in SR Ca^{2+} regulatory proteins during cardiac hypertrophy depend on the type of mechanical overload imposed (*1*, *3–6*). The mechanisms underlying these alterations are still unknown and it is unclear whether they are a direct effect or a secondary effect resulting from the imposition of mechanical overload on cardiac myocytes. In a series of *in vitro* studies, the stretching of cultured cardiac myocytes obtained from neonatal rats was shown to induce severe hypertrophy and to stimulate both protein synthesis and specific gene expression (*18*, *19*). Cadre *et al*. (*7*) reported that cyclic stretch induced hypertrophy of neonatal rat ventricular myocytes and significantly down-regulated the expressions of RyR and Ca^{2+}-ATPase (at both the mRNA and

protein levels). Such down-regulation is a characteristic result of volume-overload of the rat heart *in vivo*, as demonstrated in our previous report (*3*). The present study was designed to assess the *in vitro* effects of high ambient pressure, not of stretch-overload, on cultured neonatal cardiac myocytes and to determine whether such high ambient pressure might affect the expression of SR Ca^{2+} regulatory proteins (RyR and Ca^{2+}-ATPase) during the development of hypertrophy. Interestingly, we showed that high ambient pressure induced myocyte hypertrophy and an up-regulation of RyR and Ca^{2+}-ATPase (at both the mRNA and protein levels), effects that are characteristic of a mild pressure-overloading of the rat heart *in vivo* (*4*, *20*).

Mechanical stimuli cause a rapid change in gene expression in cardiac myocytes (*18*, *19*). Many mechanosensitive ion channels and exchangers play critical roles in stretch-induced myocyte hypertrophy (*21*). Linear stretch of cardiac myocytes *in vitro* causes a transcriptional activation of immediate-early genes, followed by an induction of the fetal genes for atrial natriuretic factor, skeletal α-actin and β-myosin heavy chain (*19*). Moreover, recent evidence indicates that mechanical stretch has a close relationship with autocrine-paracrine growth factors in the heart, such as those of the tissue renin-angiotensin system (*18*, *22*). At present, it is not clear why the expression of RyR and Ca^{2+}-ATPase proteins and genes show either an increase or a decrease depending on the type of mechanical overload. In the case of the Ca^{2+}-ATPase gene, Fisher *et al*. (*23*) demonstrated that the promoter region, which extends from the transcriptional start site to −284, produces enhanced transcriptional activity, whereas the region from −1100 to −284 exerts a negative transcriptional effect in the adult myocardium. Arai *et al*. (*20*) speculated in their report that the interaction between the *trans*-acting factors associated with hemodynamic overload and these promoter regions may be responsible for the up- or down-regulation of the Ca^{2+}-ATPase gene. Although knowledge of the cellular and molecular mechanisms underlying stretch-induced hypertrophy has increased considerably in recent years, the mechanisms underlying high ambient pressure-induced hypertrophy are still unclear and demand further investigation.

Interestingly, in our cultured neonatal rat cardiac myocytes the level of Ca^{2+}-ATPase protein gradually increased (unlike that of RyR protein) not only under HPC, but also under APC. In our experiments, we prepared cardiomyocytes from 1- or 2-day-old rat hearts and cultured then up to 7 days. Shiojima *et al*. (*24*) examined developmental changes in Ca^{2+}-ATPase and reported that the expression of Ca^{2+}-ATPase mRNA was significantly lower than the post-natal level during the early embryonic period, but increased dramatically starting 2 days before birth. These data and ours indicate that the expression of Ca^{2+}-ATPase is regulated both by hemodynamic load and development stage.

We should point out some limitations of the present study. First, in this study, a pressure of 200 mmHg was necessary to induce significant hypertrophy of the cardiomyocytes. This 200 mmHg was provided as a compressive pressure or ambient pressure onto the monolayer cells. Some may object that this value is far beyond the pressure in a real clinical setting, in which mean intraventricular pressure is usually less than 150 mmHg, even if a ventricular pressure overload exists. However, we do not know what the 200 mmHg of pressure used in our *in vitro* model corresponds to in terms of actual mean intraventricular cavity pressure, because the accurate values for the compressive pressure or compressive stress for individual cells have not been measured *in vivo*. Our study is the first report of the effect of excessive ambient pressure on myocardial cell hypertrophy, so further examination will be needed to assess the clinical applicability of our results. Second, we could not perform a functional examination of the SR, because of the difficulty of performing such studies on cultured cardiac myocytes and because the amount of SR that can be obtained from cultured cells is limited. Third, we need to monitor intracellular Ca^{2+} transients. We speculated that the increases in RyR and Ca^{2+}-ATPase proteins could cause changes in the intracellular Ca^{2+} homeostasis, but we have not directly shown that the increase in these proteins has "functional" consequences for intracellular Ca^{2+} transients. Clearly, examination of the intracellular Ca^{2+} transients of pressure-overloaded hypertrophied myocytes would be of great interest. Finally, the contraction-relaxation characteristics of cardiac myocytes subjected to high ambient pressure were regulated not only by SR Ca^{2+} regulatory proteins, but also by several other factors—*e.g.*, myofilament characteristics and/or neurohumoral factors produced by cardiomyocytes themselves. Further experimental studies will be needed to clarify the contributions made by each of these factors in this *in vitro* high pressure model.

In conclusion, our results indicate that high ambient pressure induces hypertrophy in neonatal rat cardiac myocytes and causes up-regulation of RyR and Ca^{2+}-ATPase in cultured cardiac myocytes. The results of the present study are compatible with the hypothesis that in the mechanically overloaded heart, genes encoding proteins involved in SR Ca^{2+} regulation are up- or down-regulated in a manner dependent on the type of load. The present *in vitro* high pressure model may prove useful, like stretch-overload models, for the investigation of the intracellular Ca^{2+} regulatory pathways responsible for the development of cardiac hypertrophy *in vivo*.

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