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Effect of Long-Term Hypoxia on Myocardial Alpha1- Receptors

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EFFECT OF LONG-TERM HYPOXIA ON MYOCARDIAL $ALPHA_1$- RECEPTORS

by

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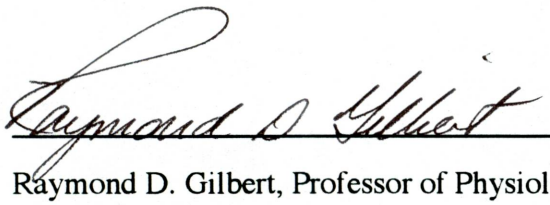

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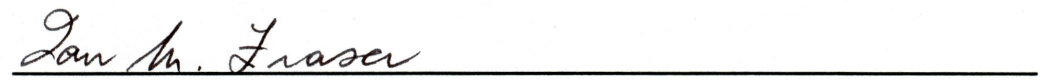

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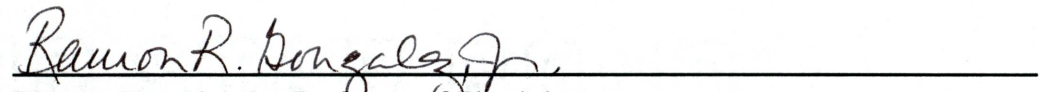

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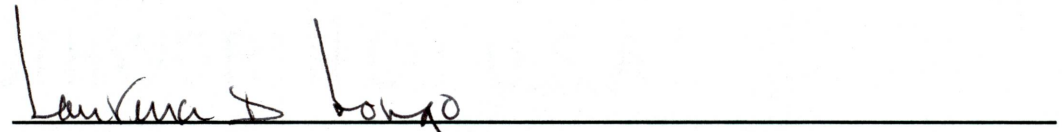

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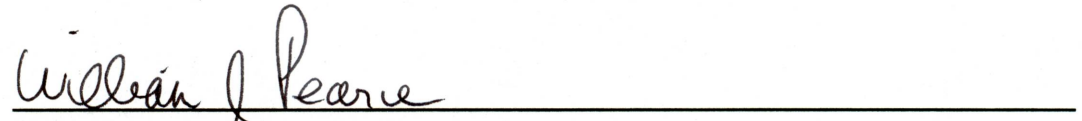

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ABSTRACT

EFFECT OF LONG-TERM HIGH-ALTITUDE HYPOXEMIA AND DEVELOPMENT ON MYOCARDIAL ALPHA$_1$-ADRENERGIC RECEPTORS AND SIGNAL TRANSDUCTION PATHWAYS

by

Bihong T. Chen

To determine the developmental changes in myocardial α_1-adrenergic receptor system, we employed [^{3}H-Prazosin] binding technique to characterize α_1-adrenergic receptors in both fetal and adult sheep myocardial ventricular membrane preparations. Myocardial α_1-adrenergic receptor density (Bmax) declined during developmental process with both fetal left ventricle(LV) and fetal right ventricle(RV) having significantly more binding sites than respective adult ventricles. We also demonstrated that the positive inotropic response to the α_1-adrenergic agonist phenylephrine did not differ significantly between the fetal and adult ventricles.

The present study also investigated the effect of long-term high-altitude hypoxemia on the α_1- and β_1-adrenergic receptor interaction in the heart. The interactions between two receptor systems were examined by prior stimulation of muscles with α_1- adrenergic agonist phenylephrine (PHE). Hypoxic left ventricle without prior phenylephrine stimulation produced greater response to isoproterenol stimulation {maximal tension developed (Tmax, g/mm^2 =: 1.16 ± 0.18)}; than normoxic left ventricle without prior phenylephrine stimulation {maximal tension developed (Tmax, g/mm^2 =: 0.41 ± 0.05)} ($p<0.01$). Myocardial α_1- and β_1- adrenergic antagonistic interaction was observed only in hypoxic LV. In conclusion, chronic hypoxemia augmented isoproterenol dose-responses

in hypoxic left ventricle. Phenylephrine antagonized effects of isoproterenol in hypoxic left ventricle indicating an α_1- and β_1- adrenergic interaction during long-term high-altitude hypoxemia.

To determine the effects of long-term high-altitude hypoxemia on myocardial α_1-adrenergic receptor system and Ins(1,4,5)P_3 (IP_3) responses, we employed [^{3}H-Prazosin] binding technique and Ins(1,4,5)P_3 assay to characterize α_1-adrenergic receptors and Ins(1,4,5)P_3 in the sheep myocardial preparations. Long-term high-altitude hypoxemia significantly depressed myocardial α_1-adrenergic receptor density (Bmax in fmol/mg) in fetal right ventricle (RV). In contrast, it did not affect the dissociation constant (Kd). Long-term hypoxemia also significantly decreased Ins(1,4,5)P_3 production in response to phenylephrine stimulation in the fetal right ventricle. In conclusion, myocardial α_1-adrenergic receptor density and Ins(1,4,5)P_3 production in response to agonist stimulation in the fetal right ventricle were decreased as result of long-term high-altitude hypoxemia.

CHAPTER ONE

INTRODUCTION

Myocardial α_1- adrenergic receptors have been found in almost all mammalian species. The undisputed existence of this receptor system has been well recognized through functional contractile study of α_1- adrenergic specific agonists such as phenylephrine and methoxamine. In addition, this receptor system has been identified by specific radioligand studies and subsequent isolation and cloning of the receptor molecules. The possible mechanisms of α_1- adrenergic receptor-mediated effects in contraction, signal transduction, cellular metabolism and electromechanical coupling have been thoroughly reviewed (Endoh,1994, Terzic 1993, Fedida 1993). This overview focuses on the different characteristics of α_1- adrenergic receptors from that of β_1- adrenergic receptors, species difference and developmental changes of myocardial α_1- adrenergic receptors, and α_1- adrenergic receptor response under pathological conditions.

Myocardial α_1- adrenergic receptors and subtypes. Three components to the inotropic response to α_1- adrenergic stimulation have been documented in rat papillary muscle. They are: a transient positive inotropism, followed by a transient negative inotropism, and then continued by a sustained positive inotropism (Otani H et al., 1987). It has also been shown that the initial inotropic response to phenylephrine stimulation is diminished by inhibition of inositol phosphate formation with neomycin. Therefore, it appears that the increase of intracellular $Ins(1,4,5)P_3$ is responsible for the initial positive inotropic response. On the other hand, the sustained positive inotropism may be mediated by diacylglycerol (DAG) and Protein Kinase C (PKC). But no conclusive data is available in regard to the role of PKC on inotropism. Studies so far have generated conflicting

findings. Hartmann HA et al (1988) showed that administration of phorbol esters exerted a negative inotropic effect on cardiac myocytes. However, other investigators have found that phorbol esters exert a positive inotropic effect or could potentiate the positive inotropic response to phenylephrine (Otani H et al, 1987). More study is needed to clarify this issue.

Myocardial α_1- adrenergic receptors can be further subdivided into at least two pharmacologically distinct subtypes (Han et al.,1987). These subtypes, α_{1A} and $\alpha_{1B,}$ can be differentiated according to their sensitivity toward selective antagonists. The α_{1A} -subtype has a higher affinity for the antagonists 5-methyle-urapidil, and WB-4101. The α_{1B} -subtype is irreversibly alkylated by chlorethylclonidine (CEC). It has been shown that 20% of α_1- adrenergic receptors belong to the α_{1A} -subtype and the remaining 80% of the binding sites may correspond to the α_{1B} -subtype (Groβ & Hanft, 1988).

Evidence has shown that these two α_1- adrenergic receptor subtypes are linked to different signal transduction pathways and effector systems. It has been suggested that the α_{1A} subtype primarily promotes the influx of extracellular calcium through dihydropyridine-sensitive channels, while the α_{1B} -subtype participate in the signal transduction pathway via phosphoinositide pathway (Minneman K et al., 1988). The α_{1B} -subtype appears to be involved in positive inotropism. Molecular cloning has demonstrated at least four distinct subtypes: $\alpha_{1A,}$ $\alpha_{1B,}$ $\alpha_{1C,}$ and α_{1D} (Price DT et al., 1994). However, the relationship between cloned α_1- adrenergic receptors and pharmacologically distinct subtypes is not completely understood.

Comparison between α_1- and β_1- adrenergic receptor systems. There are distinct differences in the inotropic effects of these two receptor systems. Compared to the β_1-adrenergic response, myocardial α_1- adrenergic receptor stimulation causes a smaller

positive inotropic response, which takes much longer time to develop. It has been shown that α_1- adrenergic stimulation can cause a positive inotropic effect, but which amounts to only 60% of the response to β_1- adrenergic stimulation (Hescheler J et al, 1988). Also, α_1- adrenergic stimulation increases twitch duration in isolated myocardium by prolonging both the contractile and relaxation phases, in contrast to β_1- adrenergic stimulation which shortens both the contractile and relaxation phases of the contraction. Furthermore, electrophysiological studies show that myofibrillar responsiveness to Ca^{2+} mediated through α_1- and β_1- adrenergic receptors change reciprocally, with phenylephrine increasing it and isopreterenol decreasing it (Encoh & Blinks, 1988). This increased calcium sensitivity of the myofilaments is probably related to activation of Na^+/H^+ exchange and alkalinization of the sarcoplasm. By sensitizing myofilaments to Ca^{2+}, α_1- adrenergic agonists could increase contractility in the heart without increasing oxygen demand.

There are also major differences in the mechanisms for α_1- and β_1- adrenergic receptor function (Figure 1). Since the first observation that phosphoinositide metabolism (PI) was regulated by adrenergic system in the heart (Gaut & Huggins, 1966), studies have demonstrated the role of inositol phosphates in signal transduction system. It is now generally accepted that phenylephrine stimulates α_1-adrenergic receptors in the heart muscle, which in turn activates phospholipase C. This phosphoinositide-specific enzyme mediates PtdIns(4,5)P_2 hydrolysis to generate Ins(1,4,5)P_3 and diacylglycerol (DAG) (Berridge 1993). Ins(1,4,5)P_3 enhances the release of calcium from intracellular stores such as the sarcoplasmic reticulum to enhance contraction. Scholz et al (1991) demonstrated in their study with isolated electrically stimulated perfused rat heart that the increase in Ins(1,4,5)P_3 preceded the increase in force of contraction. They also showed that Ins(1,4,5)P_3 and force had similar dose-responses to phenylephrine stimulation.

Furthermore, they also showed the role of $Ins(1,4,5)P_3$ both as a second messenger and as the molecular link between α_1-adrenergic receptor stimulation and inotropic responses in cardiac muscle. Thus, their study demonstrated the causal relationship between $Ins(1,4,5)P_3$ production and positive inotropism.

As shown in Figure 1, stimulation of α_1-adrenergic receptors may increase the sensitivity of the contractile proteins for Ca^{2+} (Pathway 1). This is probably related to activation of Na^+/H^+ exchange and alkalinization of the sarcoplasm. In addition, α_1-adrenergic receptor stimulation activates Phospholipase C. This phosphoinositide-specific enzyme mediates $PtdIns(4,5)P_2$ hydrolysis to generate $Ins(1,4,5)P_3$ and diacylglycerol (DAG). $Ins(1,4,5)P_3$ releases calcium from intracellular stores, such as sarcoplasmic reticulum, to enhance contraction (Pathway 2). Furthermore, α_1-adrenergic receptor activation may modulate β_1- adrenergic receptor system response (Pathway 3). In isolated rat heart stimulated with norepinephrine, the α_1-adrenergic receptor-mediated component contributes about 25 % of the final inotropic effect, whereas the β- adrenergic receptor component accounts for 75% (Skomedal T et al., 1988). Although it is not easy to observe inotropic response to α_1-adrenergic stimulation in vivo due to the predominant β- adrenergic effect, sympathomimetic amines including adrenaline and norepinephrine have been shown to induce positive inotropic effects via activation of α_1-adrenergic receptors in the low concentration range, and via the β- adrenergic receptors in the higher concentration range (Endoh M, 1991). There are both antagonistic and synergistic interactions between the α_1- and β- adrenergic receptor systems. Barrett S et al (1993) showed that α_1-adrenergic receptor stimulation inhibited accumulation of cellular cAMP in rat cardiac myocytes, due to activation of cAMP-phosphodiesterase. Because cAMP is the second messenger modulating β- adrenergic receptor activation, reduced cAMP production results in inhibition of β- adrenergic receptor function. This antagonistic

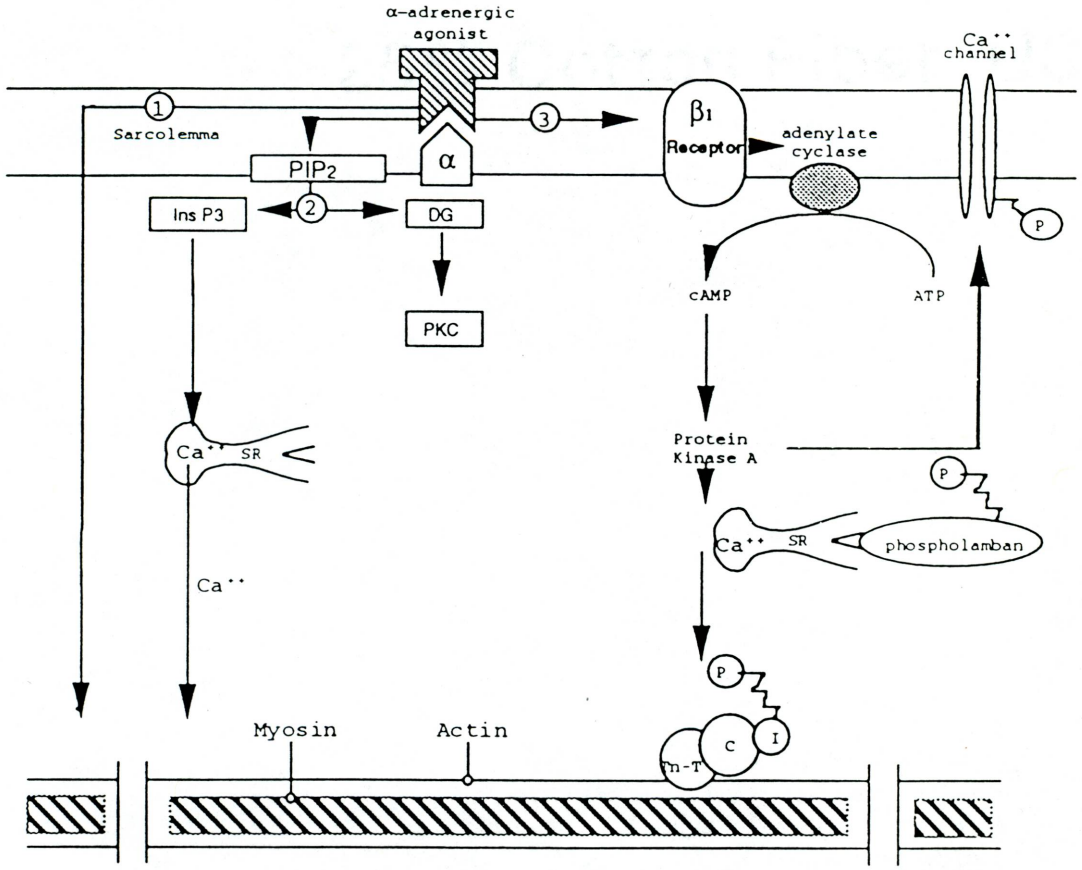

Figure 1: Activation of α-Adrenoceptors and Its Possible Role in Modulating the Effects of β-Adrenoceptor System in Mammalian Cardiac Muscle.

interaction is further supported by direct contractile studies of whole heart preparations (Osnes JB et al., 1989, Skomedal T et al., 1988). These studies indicated a mutual attenuation of the function of both α_1- and β- adrenergic receptor systems in the heart, in that β- adrenergic stimulation reduced the α_1- adrenergic effect by about 50%, while α_1- adrenergic stimulation attenuated the β- adrenergic effect by about 20-25%. On the other hand, there are studies demonstrating synergism between these two receptor systems. Butterfield MC et al (1993) showed that α_1- adrenergic receptor mediated responses were potentiated following chronic β- adrenergic stimulation in the rat heart. Contradictory findings regarding α_1- and β- adrenergic receptor interaction has prompted more vigorous study in this field.

Species differences and developmental changes in myocardial α_1-adrenergic receptors. Both the density and inotropism of α_1-adrenergic receptors vary with species and age. Rat and rabbit myocardium possess a high density of α_1-adrenergic receptor binding sites (167 and 191 fmol/mg protein, respectively) when compared with dog, and feline heart (5, and 15 fmol/mg protein, respectively) (Mukherjee et al., 1983). Developmental changes in the density of myocardial α_1-adrenergic receptors were observed in rabbit, rat and dog hearts (Nakanishi et al., 1989). In all the species studied, α_1-adrenergic receptor density in the newborn was greater than that found in the adult. Using [^{125}I]IBE2254 as a ligand, del Balso et al. (1990) found 220 fmol/mg α_1-adrenergic receptors in 1 month-old dogs versus 23 fmol/mg in the adult. This decline in receptor density may be due to diminished levels of α_1-adrenergic receptor mRNA as determined by Northern blot analysis in the aging myocardium (Kimball et al., 1991). No age-related difference in receptor affinity have been described.

There is also an apparent dissociation of receptor density and inotropism with development in myocardial α_1-adrenergic receptors. In rat, rabbit and dog heart, myocardial α_1-adrenergic receptor density decreases while positive inotropism to agonist stimulation increases with development. This could be due to the developmental differences in subcellular organelles and the calcium releasing effect of $Ins(1,4,5)P_3$. The myocardium of newborn rabbit has been shown to be less sensitive to caffeine, an activator of Ca^{2+} release from the sarcoplasmic reticulum (George BL et al., 1984). In addition, Nakanishi et al (1984) showed that the sarcoplasmic reticulum was underdeveloped in the premature heart. Therefore, it is possible that the calcium releasing effect of inositol triphosphate from this intracellular organelle and the resulting positive inotropic effect may be reduced in the young myocardium.

Myocardial α_1-adrenergic receptors under pathological conditions. It has been proposed that α_1-adrenergic receptors might serve as a reserve mechanism to maintain myocardial responsiveness to catecholamine under conditions in which the β- adrenergic receptors are not functional (Osnes et al., 1985). Studies have shown that α_1-adrenergic receptors are involved in cardiac dysfuntions, such as cardiac ischemia, cardiac hypertrophy and cardiac arrhythmia.

Myocardial α_1-adrenergic activation increases the automaticity of latent pacemakers and therefore enhances the genesis of specific arrhythmia (Sheridan, 1986). During myocardial ischemia and reperfusion, α_1-adrenergic receptors increase in numbers, which has been indicated to contribute to reperfusion-induced arrhythmia. This is supported by evidence showing that the α_1-adrenergic blocker prazosin possesses potent antiarrhythmic effects in ischemia-reperfusion damage (Kurz T et al., 1991). The mechanism for generation of arrhythmia is believed to be in the activating effect of

Na^+/H^+ antiport by α_1-adrenergic stimulation. The enhanced activity of the Na^+/H^+ antiporter results in increased intracellular Na^+ concentration, which in turn, could lead to Ca^{2+} overload by a net uptake of Ca^{2+} via the Na^+/Ca^{2+} exchange, thus contributing to generation of arrhythmia.

In addition, in simulated ischemic conditions, α_1-adrenergic receptor-mediated production of arrhythmia is significantly reduced by WB-4101, a selective α_{1A}-antagonist (Anyukhovsky and Rosen ,1991). As mention above, the α_{1A}- subtype is related to the generation of $Ins(1,4,5)P_3$, which has been observed to be significantly increased immediately after reperfusion (Mouton R et al., 1991). Therefore, it is reasonable to infer that $Ins(1,4,5)P_3$ also contribute to generation of arrhythmias. Thus, $Ins(1,4,5)P_3$ may play a role as a second messenger in ischemia/reperfusion-induced arrhythmia.

Myocardial α_1-adrenergic receptors also play a role in cardiac hypertrophy as seen in hypertension and myocardial infarction. Norepinephrine has been shown to increase protein synthesis in cultured rat myocytes (Simpson 1986). Furthermore, α_1-adrenergic activation activate the expression of protooncogenes, such as *c-myc* and *c-fos*, which may be useful markers for cardiac diseases (Thorburn et al, 1994).

Hypoxia has been shown to increase myocardial α_1-adrenergic receptor density in neonatal rat ventricular myocytes (Kagiya T et al., 1991). However, others (Steinberg et al 1993) have shown that the density of α_1-adrenergic receptor was similar in normoxic and hypoxic cultured myocytes. Steinberg et al also showed that hypoxia stimulates production of inositol phosphates by stimulation of the α_{1A}-subtype. Heather et al. (1989) showed that canine myocytes exhibit an increase in the production of $Ins(1,4,5)P_3$ in response to norepinephrine production following exposure to hypoxia for 10 min. Further study on the hypoxic effect on myocardial α_1-adrenergic receptors is needed to understand the mechanism of the effect.

Previous in vivo studies in our laboratory have shown that long-term high-altitude hypoxemia significantly depressed fetal right ventricular function, with only slightly decreased left ventricular function, resulting in a reduction in cardiac output (Kamitomo et al, 1992). Right ventricular sensitivity to afterload was also decreased. However, little is known in regard to the effect of long-term hypoxemia on α_1-adrenergic receptors and the related second messenger system. Therefore, studies are proposed to further investigate myocardial α_1-adrenergic receptors in response to both long-term hypoxemia and development. Specific experiments are designed to examine (1) the dose-response relationship to an α_1-adrenergic agonist such as phenylephrine in isolated electrically driven papillary muscles from sheep myocardium, (2) the dose-response relationship to β- adrenergic agonist such as isopreterenol with or without prior stimulation of phenylephrine to demonstrate receptor interactions between these two receptor systems, (3) myocardial α_1-adrenergic receptor density and affinity, and (4) the changes of $Ins(1,4,5)P_3$ production in response to α_1-adrenergic stimulation in the papillary muscle preparations in sheep myocardium. These studies should contribute to the understanding of the mechanisms involved in modulation of cardiac adrenergic systems by both long-term hypoxemia and development.

Conclusion: In summary, myocardial α_1-adrenergic receptors have shown to have diverse effects ranging from physiologic inotropism to pathologic arrythmogenesis. $Ins(1,4,5)P_3$ and protein kinase C appear to be the second-messengers mediating signal transduction and linking α_1-adrenergic receptor activation to positive inotropism. Myocardial α_1-adrenergic receptors exhibit changes to development and hypoxia. Further study is needed to clarify the clinical implication of the myocardial α_1-adrenergic receptor system.

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CHAPTER TWO

MYOCARDIAL ALPHA$_1$-ADRENERGIC RECEPTORS IN FETAL AND ADULT SHEEP MYOCARDIUM

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[1]Student who conducted all the experiments, collected the data, and prepared the manuscript

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ABSTRACT

To determine the developmental changes in myocardial α_1-adrenergic receptor system, we employed [^{3}H-Prazosin] binding technique to characterize α_1-adrenergic receptors in both fetal and adult sheep myocardial ventricular membrane preparations. We also measured the inotropic effect of phenylephrine in the isolated electrically driven papillary muscle preparations in these animals. two groups of animals (n=6-8 in each group) were tested: (1) Fetal hearts taken from pregnant ewes kept at sea level (300 m) during the whole gestation (2) Adult hearts taken from non-pregnant adult sheep kept at sea level. Our finding indicated an age-related differences in α_1-adrenergic receptor density (Bmax in fmol/mg protein), but not in affinity (Kd in nM). Myocardial α_1-adrenergic receptor density (Bmax) declined during developmental process with both fetal left ventricle(LV) and fetal right ventricle(RV) having significantly more binding sites (22.32 ± 2.58 for LV and 29.61 ± 3.42 for RV) than respective adult ventricles (11.09 ± 1.44 for LV and 10.29 ± 2.06 for RV). There is no ventricular difference in neither Bmax nor Kd. We also demonstrated that the positive inotropic response to the α_1-adrenergic agonist phenylephrine did not differ significantly between the fetal and adult ventricles. However, when comparing left ventricle with right ventricle either in the adult or in the fetuses, we found that the right ventricle had much more pronounced response to phenylephrine stimulation than the left ventricle. We speculate that changes in myocardial α_1-adrenergic receptor system might be due to modifications in α_1-adrenergic receptor subtypes, with resultant changes downstream in the inositol-1,4,5-triphosphate and Protein Kinase C pathways. In conclusion, myocardial α_1-adrenergic receptor density was decreased as result of development. The positive inotropic response to phenylephrine stimulation did not change with increasing age.

Key words: inotropism, phenylephrine, receptor density

INTRODUCTION

Since the first observation of the existence of α_1-adrenergic receptors in the mammalian heart by Wenzel and Su in 1966 (35), many studies have investigated the important role of this receptor system on myocardial contractility and signal transduction pathways. These studies provide insight into the possible mechanism by identifying molecular mediators such as a Gh protein and a novel transcription factor linking α_1-adrenergic receptors to a phosphalipase c (1,10). Previous studies (5,9,17,32) imply not only the presence of α_1-adrenergic receptors on cardiac muscle of various species, but also demonstrate that they are coupled to positive inotropic effects and second messenger systems such as Ins-1,4,5-Triphosphate and Protein Kinase C.

The myocardial α_1-adrenergic receptor number varies with species and changes during the developmental process with greater density in the newborn than in the adult (4,8). Nakanishi et al (22) showed that myocardial alpha-receptor density decreases and the positive inotropic effect of the alpha-agonist increases with development. They also demonstrated the role of Protein Kinase C in the reduced inotropy of alpha-agents in the newborn animals. In contrast, Endoh et al (8) demonstrated characteristic α_1-adrenergic receptor binding sites in the dog heart but failed to show any positive inotropic response to activation of the receptors.

Very few studies have been done on the α_1-adrenergic receptor system in the fetal heart. Little is known regarding the effect of development on myocardial α_1-adrenergic receptors and agonist inotropic effect in the sheep myocardium. The present study was designed to investigate (1) the inotropic effect of α_1-adrenergic stimulation by phenylephrine in the isolated papillary muscle preparation of developing sheep myocardium, and (2) the changes of myocardial α_1-adrenergic receptor density and affinity in both near-term fetuses and adults.

MATERIALS AND METHODS

Animal Preparation: Time-dated pregnant (fetus) and non-pregnant (adults) sheep were used in this study and were from a single supplier (Nebeker Ranch, Lancaster, CA). When the pregnant sheep were near-term (140-142 days), animals were transported to our laboratory at Loma Linda University Medical Center. On the experimental days, the pregnant ewes were anesthetized by an intravenous injection of thiopental (10 mg/kg) and then were ventilated with halothane 5% in oxygen. The fetuses were delivered through midline laparotomy. The hearts were excised from chest cavity and used for either a mechanical functional study or biochemical determination.

Contractile study: Both left and right ventricles were opened and papillary muscles were identified and cut into thin strips (0.8-1.0 mm in diameter and 10 mm in length) under a dissection microscope. Small loops of fine suture were tied around each end of the muscle strips. They were then dissected free and mounted vertically between a fixed hook and a force transducer (Grass Inst., Model FT 03, Qincy, MA) in 8 ml muscle baths continuously bubbled with 95% O_2 and 5% CO_2. The muscle strips were continuously superfused with Tyrode solution at 35°C and were stimulated via two longitudinal platinum electrodes at a frequency of 0.8 Hz (Grass Inst., model S/88) using 7 ms square-wave pulses at a stimulation voltage of approximately 40-60 V. Muscle contractions were recorded on an eight-channel polygraph (Gould Electronics Model RS 3800, Cleveland, OH) and stored on an on-line computer by using Real Time Data Acquisition (RTD) software developed in our laboratory (6). The following parameters of mechanical function were monitored continuously: Tmax (maximal active tension, g/mm^2), +dT/dt (rate of contraction, $g/mm^2/sec.$) and -dT/dt (rate of relaxation, $g/mm^2/sec.$). Muscle

contractions were normalized to the cross-sectional area and standardized to percent of baseline.

Papillary muscles strips were allowed to stabilize for at least one hour in 35°C Tyrode solution containing (in mM): 2.0 $CaCl_2$, 140 NaCl, 20 $NaHCO_3$, 6 KCl, 1 $MgCl_2$, 10 glucose and 5 HEPES (N-2-hydrosyethylpiperazine-N'-2 ethanesulfonic acid), pH = 7.4, until mechanical stabilization was achieved. All the experiments were performed at Lmax, the muscle length at which the development of active tension was maximal. Muscle strips were incubated in 10^{-6} M propranolol for 30 min. to block β-adrenergic receptors. Then the muscles were stimulated with phenylephrine in one log unit increments.

Binding Study: Cardiac membrane preparation was done by a modification of Karliner et al (13). The muscle strips were suspended and minced in 10 volumes of ice-cold homogenization buffer (50 mM Tris with 1 mM EDTA, pH=7.4, at 4 °C). The muscles were homogenized in a Polytron homogenizer at a setting of 5 for 10 s, three times total with at least 1 min. cooling period in between. Then they were centrifuged at 600xg at 4 °C for 15 min. to eliminate tissue debris. The supernatant from the low speed centrifugation was centrifuged at 39,100 xg at 4 °C for 30 min. The final pellet was resuspended in 1 ml ice-cold incubation buffer (50 mM Tris, 4 mM Mg^{2+} , pH=7.4). Protein content was determined using the methods of Lowry, et al (16).

The binding assay was done in duplicate with ^{3}H-Prazosin (specific activity 72.2 Ci/mmol, NET-823 PRAZOSIN[7-Methoxy-^{3}H]- New England Nuclear) as a ligand to characterize the α_1-adrenergic receptors in the presence (total binding) and absence (nonspecific binding) of 10 μM phentolamine. The final volume of the incubation medium was 250 μl containing 150 μl of membrane suspension, 40 μl of eight increasing

concentrations of ^{3}H-Prazosin (0.05 - 2.5 nM) and 50 µl or 60 µl incubation buffer with and without 10 µl phentolamine. The maximal number of α_1-adrenergic receptor binding sites (Bmax) and the dissociation constant (Kd) were determined using a non-linear regression one-site binding isotherm and Scatchard analysis.

Materials: Phenylephrine, phentolamine and propranolol were obtained from Sigma Chemicals (St. Louis, MO) and were of reagent grade. [^{3}H-Prazosin] (78.0 Ci/mmol) was purchased from DuPont-NEN (Boston, MA).

Statistical Analysis: Results were expressed as means ± SEM. Statistical significance between the groups was determined by One-way Analysis of Variance with Student-Newman-Keul's multiple comparison test. A p value less than 0.05 was considered an indication of statistical significance.

RESULTS

Figure 1 shows the contractile response to phenylephrine. In the adult left ventricle (Panel A), phenylephrine increased maximal tension (Tmax) in a dose-dependent manner to a maximum of 151 ± 7% of baseline. Although the fetal left ventricular response was slightly lower (137 ± 9%), it was not significantly so. In the adult right ventricle (Panel B), the response to phynelephrine was more robust, reaching a maximum of Tmax (218 ± 19%) of baseline. Again the fetal right ventricular response (194 ± 20%) was not different than that of the adult. Responses to phenylephrine in the rate of contraction (+dT/dt) and rate of relaxation (-dT/dt) were similar to that seen for maximal tension (Tmax). The negative log concentration at which half maximal response was achieved, i.e. (-LogEC50), did not differ significantly among these groups. Average values were:

adult left ventricle (5.22 ± 0.10), adult right ventricle (5.10 ± 0.02), fetal left ventricle (5.03 ± 0.04) and fetal right ventricle (4.73 ± 0.08).

Representative saturation binding curves of ^{3}H-Prazosin are shown in Figure 2. Table 1 shows the comparison of receptor density (Bmax) and dissociation constant (Kd) among all the groups. Bmax in the fetuses was significantly greater than in the adults and this difference was present in both ventricles. There were also age-related differences in dissociation constant (Kd). Comparing left ventricle (LV) with right ventricle (RV) in both fetuses and adults, no significant changes were detected in Bmax or Kd (Table 1).

DISCUSSION

The major findings in the present study were that α_1-adrenergic receptor density decrease with development, but positive inotropic response to phenylephrine stimulation did not differ significantly between the fatal and adult myocardium. We also demonstrated that the right ventricle in both fetus and adult had a much greater response to phenylephrine than the left ventricle.

Our results from the α_1-adrenergic receptor binding experiments are in agreement with previous studies by other investigators. The values we found for Bmax in adult left ventricle (11.09 ± 1.44 fmol/mg protein) and right ventricle (10.29 ± 2.06 fmol/mg protein) (Table 1) compare favorably to values reported previously for adult rabbit ventricle (8.3 ± 1.1 fmol/mg protein) and adult dog (13.0 ± 1.0 fmol/mg protein) (31). However, adult rat ventricle Bmax has been reported to be extremely high (67.0 ± 5.0 fmol/mg protein) (18,25).

Our finding of a much higher number of α_1-adrenergic receptor binding sites in the fetus than the adult (Table 1), is also in agreement with previous literature. Cheny et al. (5) has shown that α_1-adrenergic receptor density in the sheep declined with development

from fetus to neonate. Although species differences exist among dogs, rats, man and sheep with regard to the effects of development on α_1-adrenergic receptor density, most species are in accordance with the trend showing declined α_1-adrenergic receptor density with development (4,23). Kimball et al (15) have shown in the rat cultured myocytes that the level of α_1-adrenergic receptor mRNA as determined by northern blot analysis decreased with age. Other have shown that in the mouse (31,32) and rat (22,34) the receptor density increases during fetal life, reaches a peak in the neonate and then declines in the adult to level equivalent to those observed for the fetus.

Our finding of a positive inotropic response to α_1-adrenergic receptor stimulation in both the fetus and adult is in agreement with similar findings by others (8,9,15,17,22). We also observed that the maximal response to phenylephrine (Figure 1) did not change with development inspite of a decrease in receptor density, in agreement with the findings of Nakanishi et al (22). In addition, we demonstrated that the right ventricle had a greater inotropic response to phenylephrine stimulation than the left ventricle in both fetal or adult myocardium. At present, we have no explanation for this ventricular difference.

The apparent age-related increase in inotropic effect of α_1-adrenergic receptor stimulation could be due to changes in the second messenger system such as Ins-1,4,5-triphosphate and Protein Kinase C in signal transduction pathway. It is well recognized that mediators such as Ins-1,4,5-triphosphate and diacylglycerol are involved in the positive inotropic effect of α_1-adrenergic stimulation (2,19,20). Myocardial α_1-adrenergic stimulation causes hydrolysis of phosphotidylinositol biphosphate to form Ins(1,4,5)P_3 which activates calcium release from intracellular stores (15,24,27,28,30). Diacylglycerol activates Protein Kinase C which is also involved in muscle contraction. Nakanishi et al (22) showed that Protein Kinase C activation produced greater negative inotropy in the developing myocardium, which may in part explain the relatively smaller

inotropic effect in relation to the much higher receptor density in the immature heart. We speculate that adult animals might have higher coupling efficiency, producing higher levels of Ins-1,4,5-$P_{3,}$ resulting in greater inotropic response in relation to the relatively lower receptor density in this age group. Nakanishi et al (21) have shown the sarcoplasmic reticulum is underdeveloped in the premature heart. Therefore, it is possible that the amount of calcium stored in sarcoplamic reticulum is less, resulting in a reduced response per number of α_1-adrenergic receptors stimulated. However, recent studies in our laboratory have demonstrated the sarcoplasmic reticulum of term fetal sheep to be as functionally mature as in the adult (3).

Simpson et al (29) showed that alpha-receptors play an important role in cardiac growth. Its role in regulating cardiac growth may be more important in the premature myocardium. This could partly explain why there should be more abundant α_1-adrenergic receptors in the fetal hearts.

It has been shown that both $alpha_{1A}$ and $alpha_{1B}$ adrenergic receptor subtypes play a role in production of the positive inotropic effect mediated by myocardial α_1-adrenergic receptors (9,14,30,33). Our present study demonstrated significant difference in Kd between fetus and adult. This indicated that myocardial α_1-adrenergic receptor subtypes were changed during development. However, we did not differentiate $alpha_{1A}$ and $alpha_{1B}$ subtypes in this study. The actual changes that occur in the signal transduction pathway, in response to alterations in α_1-adrenergic receptors and the receptor subtypes during developmental process, remain to be elucidated.

In conclusion, myocardial α_1-adrenergic receptor density decreased with development from the fetus to the adult. There was also developmental difference in regard to dissociation constant. However, the inotropic response to phenylephrine stimulation did not alter significantly with development. We speculate, changes in the coupling process

subsequent to receptor stimulation together with alterations in signal transduction pathway, may be crucial in explaining the developmental difference in myocardial α_1-adrenergic receptor system.

ACKNOWLEDGMENTS: This work was supported by NICHD grant HD-22190.

FIGURE LEGENDS

Figure 1. Phenylephrine dose-response curves in sheep myocardium. Panel A: left ventricle (LV); Panel B: right ventricle (RV). Abbr.: Adult (A), Fetus(F). (n=6).

Figure 2. [^{3}H-Prazosin] saturation binding curves to the membranes derived from sheep myocardium. Receptor density (Bmax) and dissociation constant (Kd) were calculated from each binding curve for each experiment. Values presented are means of duplicate determinations in a single representative experiment. (n=6-8). Panel A: representative saturation binding curves from the left ventricular (LV) myocardium. Panel B: representative binding curves from the right ventricular (RV) myocardium.

Table 1. The maximal number of binding sites (Bmax, fmol/mg) and the dissociation constant (Kd, nM) calculated from 3H-Prazocin binding study of alpha-adrenergic receptors in the ovine cardiac membrane fractions.

		Bmax (fmol/mg)	Kd (nM)
Fetal LV	CON	22.32±2.58	0.26±0.05
	HYP	17.22±1.16	0.22±0.06
Adult LV	CON	11.09±1.44†	0.12±0.02
	HYP	6.55±1.22*	0.21±0.10
Fetal RV	CON	29.61±3.42	0.42±0.13
	HYP	18.73±2.05*	0.22±0.05
Adult RV	CON	10.29±2.06†	0.14±0.03
	HYP	6.03±1.46*	0.21±0.08

Values are mean ± SE, n = 6-8. Left Ventricle (LV), Right Ventricle (RV), Control (CON), Hypoxic (HYP). * - significantly different from control value. † - significantly different from respective fetal LV or RV control value.

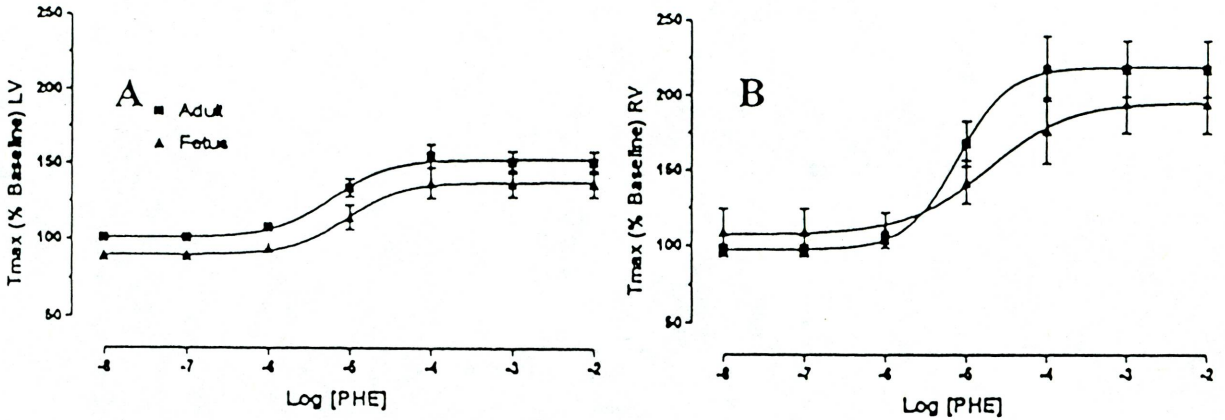

Figure 1. Phenylephrine (PHE) dose-response curves in ovine papillary muscle preparations. Panel A: left ventrile (LV); Panel B: right ventricle (RV).

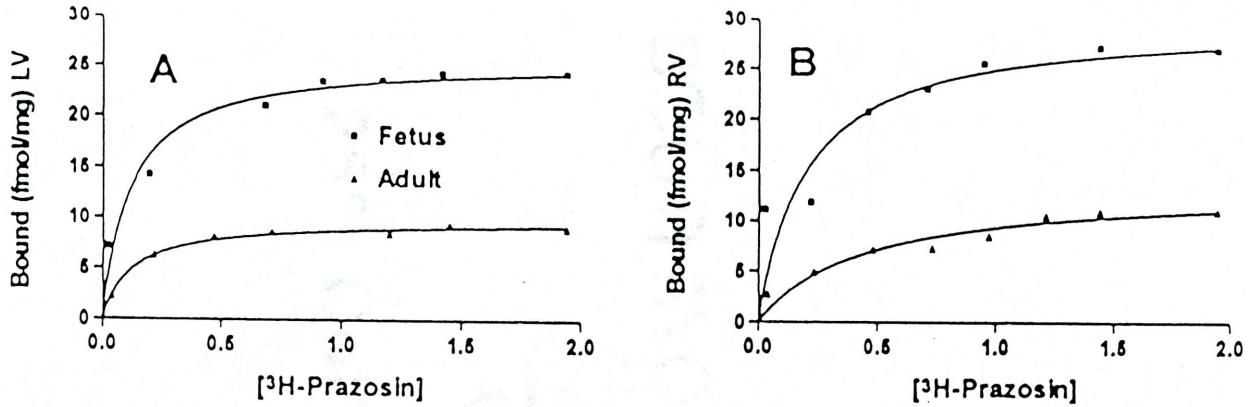

Figure 2. [^{3}H-Prazocin] saturation binding curves in ovine myocardium. Panel A: left ventricle (LV); Panel B: right ventricle (RV).

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CHAPTER THREE

EFFECT OF ALPHA$_1$-ADRENERGIC RECEPTOR ACTIVATION ON BETA$_1$-ADRENERGIC RESPONSE IN FETAL OVINE PAPILLARY MUSCLES FOLLOWING LONG-TERM, HIGH-ALTITUDE HYPOXEMIA

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[1]Student who conducted all the experiments, collected the data, and prepared the manuscript

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ABSTRACT

The present study investigated the effect of long-term high-altitude hypoxemia on the α_1- and β_1-adrenergic receptor interaction in the heart. The contractility of left ventricular (LV) and right ventricular (RV) fetal ovine papillary muscles was assessed in both α_1-adrenergic receptor agonist phenylephrine (PHE) and β_1-adrenergic agonist isoproterenol (ISO) dose response studies. The interactions between two receptor systems were examined by prior stimulation of muscles with PHE. Two groups of animals were tested: (1) hypoxic animals kept at high altitude (3,820 m) from 30 days of gestation to near term, and (2) normoxic animals kept at sea level Hypoxic left ventricle without prior PHE stimulation produced greater response to ISO stimulation {maximal tension developed (Tmax, g/mm^2): 1.16 ± 0.18; rate of contraction (+dT/dt, g/mm^2/sec.): 10.23 ± 0.89 and rate of relaxation (-dT/dt, g/mm^2/sec.): 9.28 ± 1.50)} than normoxic left ventricle without prior PHE stimulation (0.41 ± 0.05, 3.94 ± 0.71 and 3.18 ± 0.47, respectively) ($p<0.01$). In right ventricle without prior PHE treatment, only rate of relaxation (-dT/dt) was significantly modified by hypoxia with (7.61 ± 2.39) in hypoxic RV and (3.99 ± 1.15) in normoxic RV. Myocardial α_1- and β_1- adrenergic antagonistic interaction was observed only in hypoxic LV; hypoxic LV without prior PHE stimulation had higher contractility (Tmax: 1.16 ± 0.18; +dT/dt: 10.23 ± 0.89 ; -dT/dt: 9.28 ± 1.50) than hypoxic LV with prior PHE stimulation (0.30 ± 0.06, 5.15 ± 1.01 and 3.67 ± 0.47, respectively)($p<0.001$). No significant difference regarding receptor interaction was found in the right ventricle. In conclusion, chronic hypoxemia augmented ISO dose-responses in hypoxic left ventricle. PHE antagonized effects of ISO in hypoxic left ventricle indicating an α_1- and β_1- adrenergic interaction during long-term high-altitude hypoxemia.

Key words: inotropism, receptor interaction, dose-response study

INTRODUCTION

Many studies investigating α_1- and β_1- adrenergic receptor interaction in the heart have demonstrated both antagonistic and synergistic interactions between these two receptor systems. Barrett et al (3) showed that α_1- adrenergic receptor stimulation inhibited accumulation of cellular cAMP in rat cardiac myocytes. Since cAMP is the second messenger modulating contractility from β_1- adrenergic receptor activation, reduced cAMP production resulted in inhibition of the β_1- adrenergic response. This antagonistic interaction is further supported by direct contractile studies of whole heart preparations (26,28). These studies indicated a mutual attenuation of the function of both α_1- and β_1- adrenergic receptor systems in the heart, in that β_1- adrenergic stimulation reduced the α_1- adrenergic effect by about 50 %, while α_1- adrenergic stimulation attenuated the β_1- adrenergic effect to a lesser degree (about 20-25%). On the other hand, there are studies demonstrating synergism between these two receptor systems. Butterfield et al (6) showed that α_1- adrenergic receptor mediated responses were potentiated following chronic β_1- adrenergic stimulation in the rat heart. Contradictory findings regarding α_1- and β_1- adrenergic receptor interaction has prompted more vigorous study in this field.

In the present study, we used phenylephrine as an α_1- adrenergic receptor agonist and isoproterenol as a β_1-adrenergic agonist to test the interaction between α_1- and β_1- adrenergic systems in the heart. We conducted isoproterenol dose-response studies with or without prior stimulation with phenylephrine in the fetal ovine papillary muscles. The purposes of this study were: (1) to investigate the effect of α_1- adrenergic stimulation on contractility in the fetal heart, (2) to determine the effects of long-term high-altitude hypoxemia on α_1- adrenergic stimulation, and (3) to determine the effects of α_1-

adrenergic stimulation on the β_1- adrenergic response in both normoxic and hypoxic fetuses.

MATERIALS AND METHODS

Pregnant sheep (n=6) were transported to a high-altitude research station (Barcroft Laboratory, White Mountain Research Station, WMRS, Bishop, CA; elevation 3,820 m, barometric pressure, 480 Torr) at 30 days of gestation, where they remained until near term (term = 147 days). Another group of pregnant sheep (n=6), which served as normoxic controls, were kept at low altitude (300 m) throughout gestation. All the animals used in this study were time-dated and were obtained from a single supplier (Nebeker Ranch, Lancaster, CA). At 140-142 days of gestation, both groups were transported to our laboratory at Loma Linda University Medical Center, where the hypoxic group was maintained hypoxic (PaO_2 = 60 Torr) by nitrogen flow through a non-occlusive tracheal catheter. On the experimental day, the pregnant ewes were anesthetized by an intravenous injection of thiopental (10 mg/Kg) and were ventilated with 5% halothane in oxygen. The fetus was delivered through midline laparotomy, and the heart was rapidly removed and transferred to a dissection dish containing heparinized Tyrode solution continuously bubbled with 95% O_2 and 5% CO_2.

Both left and right ventricles were opened and papillary muscles were identified and cut into thin strips (0.8-1.0 mm in diameter and 10 mm in length). Small loops of fine suture were tied around each end of the muscle strips. The muscle strips were dissected free and mounted vertically between a fixed hook and a force transducer (Grass Inst., Nodel FT 03, Qincy, MA) in an 8 ml muscle bath continuously bubbled with 95% O_2 and 5% CO_2. The muscle strips were continuously superfused with Tyrode solution at 35 °C and were stimulated via two longitudinal platinum electrodes at a frequency of 0.8

Hz (Grass Inst., model S/88) using 7 ms square-wave pulses at a stimulation voltage of approximately 40-60 V. Muscle contractions were recorded on an eight-channel polygraph (Gould Electronics Model RS 3800, Cleveland, OH) and stored on computer using software developed in our laboratory (12). Three parameters were measured: maximal active tension (Tmax), peak rate of contraction (+dT/dt) and peak rate of relaxation (-dT/dt). They were normalized by the cross-sectional area of each muscle strip and standardized to percent of baseline.

The muscles were allowed to stabilize for at least one hour in 35°C Tyrode solution containing (in mM): 2.0 $CaCl_2$, 140 NaCl, 20 $NaHCO_3$, 6 KCl, 1 $MgCl_2$, 10 glucose and 5 HEPES (N-2-hydrosyethylpiperazine-N'-2 ethanesulfonic acid), pH = 7.4. All the experiments were performed at Lmax, the muscle length at which the development of active tension was maximal. The dose-response study to phenylephrine was conducted in the continuous presence of 1 μM of propranolol to block β_1- adrenergic receptors. Dose-response studies were done in a cumulative manner by increasing the concentration of agonist in log units. The drug concentrations producing 50% of the maximal response (EC50) were calculated from these dose-response curves. Isoproterenol dose-response studies were conducted with or without prior 10 μM phenylephrine stimulation. 10 μM phenylephrine produced about 50% of the maximal response.

All chemicals were purchased from Sigma Chemicals, St. Louise, MO. and of regent grade.

Statistical analysis was done using one-way Analysis of Variance. Post hoc testing of the analysis of variance was done using Duncan's Multiple Comparison Test. A p value less than 0.05 was considered an indication of statistical significance. All data

were presented as means ± SEM. Values obtained from duplicates of muscle strips from the same animal were averaged and counted as a single determination.

RESULTS

Phenylephrine dose-response study: Figure 1 shows the phenylephrine dose-response relationship for maximal tension (Tmax) in normoxic, control fetal ovine papillary muscle preparations. Phenylephrine produced a positive inotropic response in both ventricles resulting in an augmentation of Tmax to 137.14 ± 9.05 % of baseline in the left ventricle and a significantly greater augmentation ($p<0.05$) in the right ventricle (194.46 ± 19.97). However, the pD_2 values (-LogEC50) were similar for the left ventricle (5.03 ± 0.04) and the right (4.73 ± 0.08). A phenylephrine concentration of 10 μM was identified as the appropriate dose (around EC50) to be used in the subsequent experiments on receptor interaction. Both rate of contraction (+dT/dt) and rate of relaxation (-dT/dt) had dose-response curves similar to those of Tmax.

Isoproterenol dose-response study: Isoproterenol stimulation alone increased Tmax (Figure 2) in both the normoxic and hypoxic left ventricle, with the increase being greater in the hypoxic left ventricle (Table 1). Findings for +dT/dt and -dT/dt were the same as for Tmax (Table 1). In the right ventricle, isoproterenol alone augmented Tmax in both normoxic and hypoxic right ventricle (Figure 2). Although the response of the hypoxic right ventricle tended to be greater than that in the normoxic right ventricle, the difference was significant for only -dT/dt (Table 1).

Left and right ventricles exposed to long-term hypoxemia had significantly lower EC50 values in response to isoproterenol when compared to the normoxic controls (Table 2), indicating that the sensitivity of papillary muscle to isoproterenol stimulation was greatly enhanced by hypoxemia.

Receptor interaction: In the normoxic left ventricle, pretreatment with 10 μM phenylephrine had no effect on its response to isoproterenol (Figure 2 and Table 1). However, in the hypoxic left ventricle, pretreatment with phenylephrine significantly attenuated the response of all parameters to isoproterenol, with resposes reduced to the level of those observed in the normoxic left ventricle. In the right ventricle, no significant effect of phenylephrine pretreatment on the isoproterenol response was observed in either normoxic or hypoxic hearts (Figure 2 and Table 1).

DISCUSSION

The major findings in this study were: (1) phenylephrine exerted a positive inotropic effect on fetal ovine papillary muscle preparations from normoic left and right ventricle, (2) the hypoxic left ventricle demonstrated a greater response in developed tension, rate of contraction and rate of relaxation than normoxic left ventricle to isoproterenol , and (3) phenylephrine attenuated the positive inotropic effect of β_1-adrenergic stimulation in the hypoxic fetal left ventricle.

Although the β_1- adrenergic receptor system is recognized to be dominant in modulating myocardial contractility (16,25), our present study demonstrates that the α_1-adrenergic receptor system is also involved in modulating contractility in fetal ventricles. Phenylephrine is believed to increase force by increasing both calcium transient and calcium sensitivity, both by a direct action on the myofilaments and also by stimulation of Na^+/H^+ exchange leading to an intracellular alkalosis (17).

Our present study also demonstrated a receptor-mediated concentration-dependent increase in contraction to agonist phenylephrine and isoproterenol stimulation in fetal ovine papillary muscle. In our study, phenylephrine stimulated muscle to various degrees (from 121.53% (in normoxic right ventricle) to 187.91% (in hypoxic left ventricle) of

baseline) in different groups. The highest isoproterenol concentration (1μM) tested in our study also stimulated muscle to various degree from 123% (in normoxic left ventricle) to 267% baseline (in hypoxic left ventricle). It implied that the two receptor systems possess different characteristics for their inotropic effects. This was supported by previous observations (8, 22). Osnes et al (26) showed that myocardial α_1-adrenergic inotropic effects were only half of the β_1 -adrenergic effects in rat and rabbit myocardium. In addition, Aoyagi et al (1) showed that α_1-adrenergic and β_1- adrenergic receptor - mediated inotropism had qualitatively different effects on the time course of contraction and energetic efficiency, in that isoproterenol shortened the time course and phenylephrine prolonged the time course. More work needs to be done to determine the mechanism responsible for the inotropic effects of both α_1 and β_1 -adrenergic stimulation.

It is generally accepted that phenylephrine stimulates α_1-adrenergic receptors in the heart, which in turn activate Phospholipase C. This phosphoinositide-specific enzyme mediates PtdIns(4,5)P_2 hydrolysis to generate Ins(1,4,5)P_3 and diacylglycerol (DAG) (2,4,11). Ins(1,4,5)P_3 enhances the release of calcium from intracellular stores such as sarcoplasmic reticulum to enhance contraction. Alpha$_1$-adrenergic receptor stimulation results in the formation of inositol phosphate in a concentration-dependent manner similar to that of the inotropic response (15,27). Our result from phenylephrine dose-response study of contraction showed a concentration-dependent increase in force generation (Figure 1). It is reasonable to infer that Ins(1,4,5)P_3 might be involved in the phenylephrine concentration-dependent increase in force generation.

Myocardial β_1- adrenergic receptors utilize cAMP dependent protein kinase mediated phosphorylation of calcium channels, troponin and phospholamban, resulting in enhanced contractility of cardiac muscle (9,13). Our study indicated that both left and right ventricles under both normoxic and hypoxic conditions had typical concentration-

dependent increases of contraction in response to isoproterenol stimulation. However, the hypoxic left ventricle demonstrated the greatest response (Figure 2 and Table 1). We speculate that long-term high-altitude hypoxemia might initiate changes at the receptor level, such as increased number of myocardial β_1- adrenergic receptors or changes distal to the receptors, such as increased level of cAMP or protein kinase.

Our data indicated that α_1-adrenergic activation antagonized β_1-adrenergic response in the hypoxic fetal left ventricle. Other investigators report similar findings in regard to the interaction of these two receptors. Buxton et al (7) showed that incubation of intact myocytes with norepinephrine leads to significantly less cyclic AMP accumulation than stimulation with either norepinephrine plus prazosin or isoproterenol, due to activation of cAMP phosphodiesterase. Other studies have also shown that agonist occupation of α_1-adrenergic receptors inhibits β_1-adrenergic stimulated cAMP accumulation, which was attributed to receptor coupling to a guanine nucleotide inhibitory protein (3,23,24). Further, electrophysiological studies have shown that myofibrillar responsiveness to Ca^{2+} is increased with phenylephrine, but decreased with isoproterenol (14).

In conclusion, our study demonstrated that phenylephrine exerted a positive inotropic effect in the fetal heart. It also showed that hypoxemia augmented isoproterenol dose-response in the left ventricle. Finally, α_1- adrenergic receptor activation exerted an antagonizing effect on β_1- adrenergic receptor system in fetal left ventricle following long-term high-altitude hypoxemia.

ACKNOWLEDGMENTS: This work was supported by NICHD grant HD-22190.

FIGURE LEGENDS

Figure 1. Phenylephrine (PHE) dose-response in normoxic, control fetal ovine papillary muscle preparations (n=6). Left Ventricle (LV), Right Ventricle (RV).

Figure 2. Isoproterenol (ISO) dose-response study in the presence and absence of phenylephrine (PHE) in left ventricle (LV) (*Panel A*) and right ventricle (RV) (*Panel B*). (PHE+ISO) indicates isoproterenol dose-response study with prior stimulation of 10 μM phenylephrine. (ISO ONLY) indicates isoproterenol dose-response study without prior stimulation of 10 μM phenylephrine.

Table 1. Tmax, +dT/dt, and -dT/dt to maximal isoproterenol stimulation in the presence (PHE+ISO) and absence (ISO ONLY) of 10 μM Phenylephrine in fetal ovine papillary muscle strips.

		Tmax (g/mm^2)	+dT/dt ($g/sec/mm^2$)	-dT/dt ($g/sec/mm^2$)
LV (ISO ONLY)	Normoxic	0.41±0.05	3.94±0.71	3.18±0.47
	Hypoxic	1.16±0.18*	10.23±0.89*	9.28±1.50*
LV (PHE+ISO)	Normoxic	0.44±0.12	3.43±0.63	3.25±0.45
	Hypoxic	0.30±0.06†	5.15±1.01†	3.67±0.47†
RV (ISO ONLY)	Normoxic	0.61±0.11	4.83±1.05	3.99±1.15
	Hypoxic	0.92±0.31	7.89±2.38	7.61±2.39*
RV (PHE+ISO)	Normoxic	0.40±0.13	2.20±0.34	1.87±0.32
	Hypoxic	0.50±0.08	4.72±0.55*	4.42±0.42

Values are mean ± SE, n = 6-8. Isoproterenol (ISO), Phenylephrine (PHE), Left Ventricle (LV), Right Ventricle (RV). * - significantly different from respective normoxic value ($p<0.05$), † - significantly different from respective LV (ISO ONLY) value ($p<0.05$).

Table 2. LogEC50 for Tmax, +dT/dt, and -dT/dt from Isoproterenol dose-response in the presence (PHE+ISO) or absence (ISO ONLY) of 10 μM Phenylephrine in fetal ovine paillary muscel strips.

		Tmax (g/mm^2)	+dT/dt ($g/sec/mm^2$)	-dT/dt ($g/sec/mm^2$)
LV (ISO ONLY)	Normoxic	-6.44±0.27	-6.53±0.26	-6.54±0.25
	Hypoxic	-7.65±0.13*	-7.76±0.04*	-7.59±0.19*
LV (PHE+ISO)	Normoxic	-6.32±0.21	-6.57±0.15	-6.51±0.14
	Hypoxic	-7.82±0.14*	-7.55±0.20*	-7.59±0.26*
RV (ISO ONLY)	Normoxic	-6.72±0.17	-6.79±0.21	-6.73±0.18
	Hypoxic	-7.42±0.21*	-7.39±0.18*	-7.50±0.35*
RV (PHE+ISO)	Normoxic	-6.75±0.14	-6.04±0.15	-6.31±0.13
	Hypoxic	-7.59±0.29*	-7.32±0.27*	-7.42±0.25*

Values are mean ± SE, n = 6-8. Isoproterenol (ISO), Phenylephrine (PHE), Left Ventricle (LV), Right Ventricle (RV).
* - significantly different from respective normoxic value ($p<0.05$).

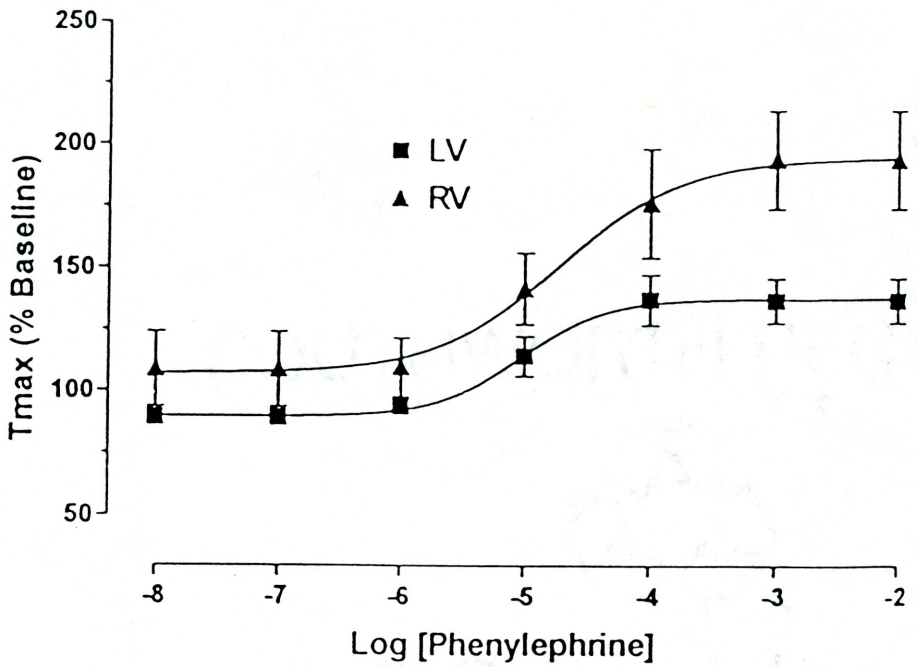

Figure 1. Phenylephrine dose-response in normoxic, control fetal ovine papillary muscle preparations (n = 6). Left Ventricle (LV), Right Ventricle (RV).

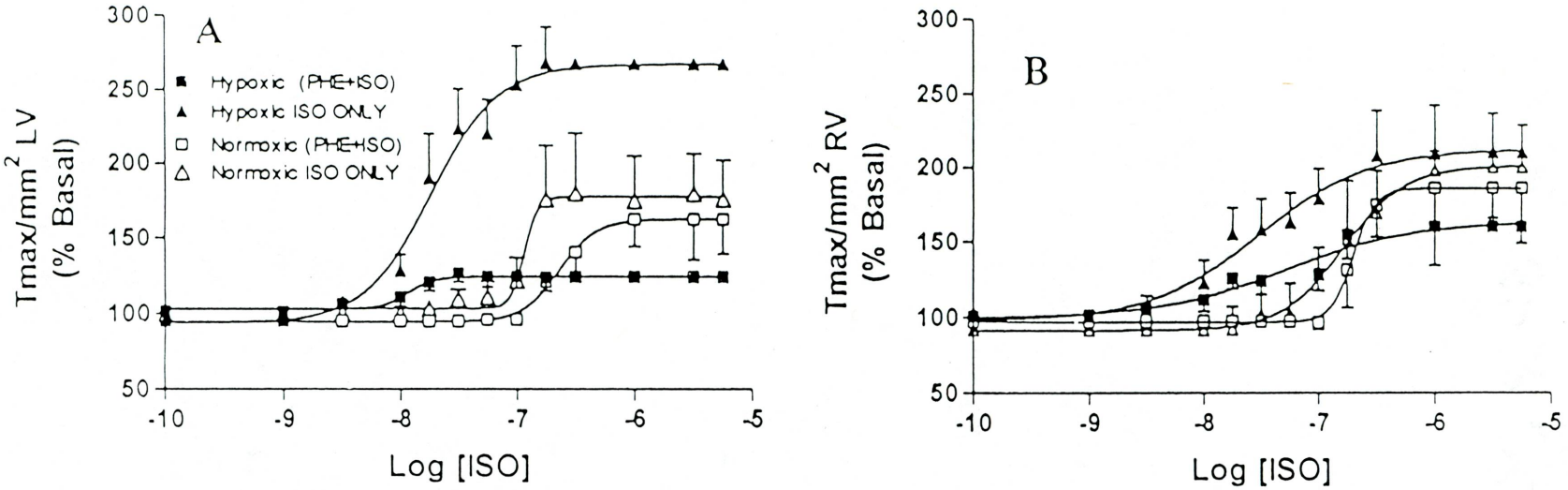

Figure 2. Isoproterenol (ISO) dose-response study in the presence and absence of phenylephrine (PHE) in the left ventricle (Panel A) and right ventricle (Panel B). (PHE+ISO) indicates prior stimulation with 10 μM phenylephrine. (ISO ONLY) indicates no prior stimulation.

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CHAPTER FOUR

EFFECT OF LONG-TERM HIGH-ALTITUDE HYPOXEMIA ON α_1-ADRENERGIC RECEPTORS AND INS(1,4,5)P_3 RESPONSES IN FETAL SHEEP MYOCARDIUM

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[1]Student who conducted all the experiments, collected the data, and prepared the manuscript

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ABSTRACT

To determine the effects of long-term high-altitude hypoxemia on myocardial α_1-adrenergic receptor system and Ins(1,4,5)P_3 (IP_3) responses, we employed [^{3}H-Prazosin] binding technique and Ins(1,4,5)P_3 assay to characterize α_1-adrenergic receptors and Ins(1,4,5)P_3 in the sheep myocardial preparations. Two groups of animals (n=6-8 in each group) were tested: (1) hypoxic fetal hearts harvested from pregnant ewes kept at high altitude (3,820 m) from 30 days of gestation to near term (140-142 days), (2) control fetal hearts taken from pregnant ewes kept at sea level (300 m) during the whole gestation period. Long-term high-altitude hypoxemia significantly depressed myocardial α_1-adrenergic receptor density (Bmax in fmol/mg) in fetal right ventricle (RV) (18.73 ± 2.05 vs. 29.61 ± 3.42). In contrast, it did not affect the dissociation constant (Kd). Long-term hypoxemia also significantly decreased Ins(1,4,5)P_3 production in response to phenylephrine stimulation in the fetal right ventricle (hypoxic RV 176.58 ± 11.31 vs normoxic RV 300.90 ± 26.95, pmol/mg protein). There is no ventricular difference in neither Bmax nor Kd. Normoxic right ventricle has the highest Ins(1,4,5)P_3 production among all the groups. In conclusion, myocardial α_1-adrenergic receptor density and Ins(1,4,5)P_3 production in response to agonist stimulation in the fetal right ventricle were decreased as result of long-term high-altitude hypoxemia. We speculate that changes in myocardial α_1-adrenergic receptor system as result of hypoxemia cause modifications in the signal transduction pathway such as Ins(1,4,5)P_3 production.

Key words: binding study, inotropism, time-course study, papillary muscle

INTRODUCTION

Since the first observation of the existence of α_1-adrenergic receptors in the mammalian heart by Wenzel and Su in 1966 (30), many studies have investigated the effect of α_1-adrenergic receptors on myocardial contractility and also the relationship between the receptor system and the second messenger system, such as Ins(1,4,5)P_3 (1,6,14). These studies prove not only the presence of α_1-adrenergic receptors on cardiac muscle of various species such as guinea pigs, rabbits, cats, lambs, monkeys and human (4,21), but also demonstrate that they are coupled to Ins(1,4,5)P_3 and to positive inotropic responses to agonist stimulation. (16,28).

Myocardial α_1-adrenergic receptor number varies from species to species and changes during the developmental process with greater density in the newborn than in the adult (3,7,19). Studies of the effects of perturbations, such as hypoxia on α_1-adrenergic receptors have been relatively sparse. Kagiya et al (9) demonstrated in rat cardiac myocytes that hypoxia significantly decreased norepinephrine binding affinity. Steinberg et al (26) have shown that the density of α_1-adrenergic receptors was similar in normoxic and hypoxic myocytes, however these studies were done in short-term (2 hr.) hypoxic conditions. Nothing is known regarding the effect of long-term hypoxemia on myocardial α_1-adrenergic receptors and Ins(1,4,5)P_3 production in the fetal heart. The present study was designed to investigate (1) the effect of long-term high-altitude hypoxemia on α_1-adrenergic receptor density and affinity in the fetal heart, and (2) the effect of long-term high-altitude hypoxemia on Ins(1,4,5)P_3 production in the fetal heart.

MATERIALS AND METHODS

Animal Preparation: Groups of pregnant sheep (n=6-8) at 30 days of gestation were transported to Bacroft Laboratory, White Mountain Research Station, WMRS (elevation 3,820 m, barometric pressure, 480 Torr). These animals were kept at the high-altitude station for 110 days. A group of pregnant sheep kept at sea level served as normoxic controls. All animals used in this study were time-dated and were from a single supplier Nebeker Ranch, Lancaster, CA. When these animals were near-term (140-142 days), they were transported to our laboratory at Loma Linda University Medical Center and maintained hypoxemic (PaO_2=60 Torr) by nitrogen flow through a non-occlusive tracheal catheter for the hypoxic group. On the experimental days, the pregnant ewes were anesthetized by an intravenous injection of thiopental (10 mg/kg) and ventilated with halothane 5% in oxygen. The fetuses were delivered through midline laparotomy, the hearts were excised from chest cavity, and free wall strips (≈ 1 gram) were cut from both left and right ventricles and frozen at -70°C for radioligand binding studies. Papillary muscles were cut into thin strips for Ins(1,4,5)P_3 studies.

Radioligand binding Study: Cardiac membrane preparation was done by a modification of Karliner, et al (12). The muscle strips were suspended and minced in 10 volumes of ice-cold homogenization buffer (50 mM Tris with 1 mM EDTA, pH=7.4, at 4 °C). The muscles were homogenized in a Polytron homogenizer at a setting of 5 for 10 s, three times total with at least 1 min. cooling period in between. Then they were centrifuged at 600xg at 4 °C for 15 min. to eliminate tissue debris. The supernatant from the low speed centrifugation was centrifuged at 39,100 xg at 4 °C for 30 min. The final pellet was resuspended in 1 ml ice-cold incubation buffer (50 mM Tris, 4 mM Mg^{2+}, pH=7.4). Protein content was determined using the methods of Lowry, et al (15).

The binding assay was done in duplicate with ^{3}H-Prazosin (specific activity 72.2 Ci/mmol, NET-823 PRAZOSIN[7-Methoxy-^{3}H]- New England Nuclear) as a ligand to characterize the α_1-adrenergic receptors in the presence (total binding) and absence (nonspecific binding) of 10 μM phentolamine. The final volume of the incubation medium was 250 μl containing 150 μl of membrane suspension, 40 μl of eight increasing concentrations of ^{3}H-Prazosin (0.05 - 2.5 nM) and 50 μl or 60 μl incubation buffer with and without phentolamine. The maximal number α_1-adrenergic receptor binding sites (Bmax) and the dissociation constant (Kd) were determined using a non-linear regression one-site binding isotherm and analysis.

Contractile study: Both left and right ventricles were opened and papillary muscles were identified and cut into thin strips (0.8-1.0 mm in diameter and 10 mm in length) under a dissection microscope. A set of eight muscle strips from the left ventricle in each hypoxic fetus were used for the simultaneous mechanical time-course study of both tension and Ins(1,4,5)P_3. Small loops of fine suture were tied around each end of the muscle strips. They were then dissected free and mounted vertically between a fixed hook and a force transducer (Grass Inst., Model FT 03, Qincy, MA) in 8 ml muscle baths continuously bubbled with 95% O_2 and 5% CO_2. The muscle strips were continuously superfused with Tyrode solution at 35°C and were stimulated via two longitudinal platinum electrodes at a frequency of 0.8 Hz (Grass Inst., model S/88) using 7 ms square-wave pulses at a stimulation voltage of approximately 40-60 V. Muscle contractions were recorded on an eight-channel polygraph (Gould Electronics Model RS 3800, Cleveland, OH) and on computer using Real Time Data Acquisition (RTD) software developed in our laboratory (5). Maximal active tension (Tmax) was measured and standardized to percent of baseline.

The muscles were allowed to stabilize for at least one hour in 35°C Tyrode solution containing (in mM): 2.0 $CaCl_2$, 140 NaCl, 20 $NaHCO_3$, 6 KCl, 1 $MgCl_2$, 10 glucose and 5 HEPES (N-2-hydrosyethylpiperazine-N'-2 ethanesulfonic acid), pH = 7.4, until mechanical stabilization was achieved. All the experiments were performed at Lmax, the muscle length at which the development of active tension was maximal. Muscle strips were incubated in 10^{-6} M propranolol for 30 min. to block β-adrenergic receptors. Then the muscles were stimulated with 10^{-4} M phenylephrine for various time (0.5 - 15 min.). At the end of each time point, the maximal tension was recorded and tissues were flash frozen in liquid N_2 for Ins(1,4,5)P_3 measurements. Some muscle strips were subjected to various phenylephrine concentrations (10^{-3}-10^{-6} M), without Ins(1,4,5)P_3 determinations, to characterize the dose-response relationship between phenylephrine and maximal active tension production.

Ins(1,4,5)P_3 test-tube study: To further test the time course of Ins(1,4,5)P_3 production following phenylephrine, thin strips of normoxic left ventricular papillary muscle were excised and placed in borosilicate glass test tubes containing 4 ml of oxygenated Tyrode solution and 10^{-6}M propranolol. The muscles were not electrically stimulated, but were at rest throughout the study. After a baseline period (30 min.), 10^{-4} M phenylephrine was added to each tube and allowed to incubate for time ranging between 30 - 180 sec., after which the tissues were flash frozen in liquid N_2 for later Ins(1,4,5)P_3 analysis.

After determination of the optimal time for peak Ins(1,4,5)P_3 production, papillary muscle strips from the normoxic right ventricle were used to determine the dose-response relation between Ins(1,4,5)P_3 and phenylephrine. The muscles were excised, placed in test tubes (without electrical stimulation), and exposed to phenylephrine in doses ranging

from 10^{-3} - 10^{-6} M. After 60 sec., the tissues were frozen in liquid N_2 for later Ins(1,4,5)P_3 determination.

Finally, after determination of the optimal time and concentration of phenylephrine exposure, Ins(1,4,5)P_3 production was measured in left and right ventricular papillary muscles from both normoxic and hypoxic fetuses. The tissues were excised, placed in test tubes, not electrically stimulated, and exposed to 100 μM phenylephrine for 60 sec. In some of the test tubes 10^{-7}M prazosin was added to determine if phenylelphrine stimulation of Ins(1,4,5)P_3 production was mediated entirely through α_1-adrenergic receptors. At the end of the exposure, tissues were frozen in liquid N_2 for later Ins(1,4,5)P_3 determination.

Measurements of Ins(1,4,5)P_3 Production: Ins(1,4,5)P_3 production in papillary muscles was measured using the DuPont-NEN [^{3}H]IP_3 assay system (Boston, MA). Briefly, frozen muscle strips were homogenized with Polytron homogenizers in ice-cold 1 M trichloroacetic acid (TCA) and then centrifuged (2,000 g) for 30 min. at 4°C. The supernatant was extracted with water saturated ether for two times. [^{3}H] inositol-1,4,5 -triphosphate was added to the extracted samples. The mixture was incubated for 60 min. at 4°C and then centrifuged (3,500 g) for 30 min. The pellet was suspended in a small amount of 0.15 M NaOH. With the addition of scintillant, the radioactivity was counted in a scintillation counter and the amount of Ins(1,4,5)P_3 was calculated from the standard curve.

Material: [^{3}H]inositol phosphate standards were obtained from DuPont-NEN (Boston, MA). Other chemicals were purchased from Sigma Chemicals, St. Louise, MO. and of regent grade. All drugs were expressed as molar concentrations in the organ bathing and test tube solutions.

Statistical Analysis: Results were expressed as means ± SEM of n preparation (n=5-8). Statistical significance of the difference between the groups was determined by One-way Analysis of Variance with Student-Newman-Keul's multiple comparison test. A p value less than 0.05 was considered an indication of statistical significance.

RESULTS

Radioligand binding study: Representative saturation binding curves of ^{3}H-Prazosin are shown in Figure 1. Actual values for Bmax and Kd are given in Table 1. In the long-term high-altitude hypoxemia group, α_1-adrenergic receptor density (Bmax) was significantly lower in the hypoxic right ventricle compared to the controls. The fetal left ventricle demonstrated a small, but insignificant reduction in Bmax. There were no hypoxemia-related differences in dissociation constant (Kd) in either left or right ventricle (Table 1).

Time-course and dose-response study: Figure 2 shows the temporal relationship between contractile force and Ins(1,4,5)P_3 production in the isolated electrically driven papillary muscle preparations from hypoxic fetal left ventricles stimulated with 100 μM phenylephrine. From a basal Ins(1,4,5)P_3 level of 9.51 ± 1.26 pmol/mg protein (n=6) Ins(1,4,5)P_3 production increased rapidly, reaching a maximum at 60 seconds, and declined thereafter to levels at or below baseline. Contractile force (tension) did not increase significantly until 2 min. and reached a maximum only after 10 - 15 minutes.

Figure 3 shows the time-course study of Ins(1,4,5)P_3 responses to phenylephrine (100 μM) stimulation in resting papillary muscles from normoxic fetal left ventricles. Similar to the electrically stimulated muscle, Ins(1,4,5)P_3 rose rapidly from a basal level

of 9.93 ± 1.63 pmol/mg protein (n=6) to reach a peak at 60 seconds. It returned to basal levels at 90 sec. and remained at low levels until 180 seconds.

Figure 4 shows the Ins(1,4,5)P_3 response to different concentrations of phenylephrine in papillary muscles from normoxic fetal right ventricles, demonstrating a maximal production of Ins(1,4,5)P_3 at 100 μM phenylephrine. Previously (see Chapter 2, Figure 1), we have demonstrated that fetal papillary muscles from normoxic fetuses achieve maximum active tension development at 100 μM (10^{-4} M) concentration. Figure 5 demonstrates that maximum active tension development in the left ventricle from hypoxic fetuses is also achieved at 100 μM (10^{-4} M) phenylephrine.

Based on these and previous data, 100 μM (10^{-4} M) phenylephrine was identified as the agonist concentration for both the maximal Ins(1,4,5)P_3 production and force generation, with maximal Ins(1,4,5)P_3 production occurring at 60 seconds. Therefore, in all subsequent experiments, 10^{-4} M phenylephrine was used to stimulate muscle strips for 60 sec.

Ins(1,4,5)P_3 study: Figure 6 shows Ins(1,4,5)P_3 production for left and right ventricles from both control and hypoxic fetuses. Table 2 shows the actual values of Ins(1,4,5)P_3 production in response to phenylephrine stimulation. The basal values for both left and right ventricles in the normoxic fetuses were 10.48 ± 1.24 and 11.63 ± 1.50 pmol/mg protein (n=6 for each group). The basal values for both hypoxic left and right ventricles were 9.90 ± 2.10 and 11.38 ± 2.06 pmol/mg protein (n=5 for each group). There were no significant differences in basal Ins(1,4,5)P_3 production among the groups. Phenylephrine stimulated Ins(1,4,5)P_3 in the normoxic right ventricle to about 300% of basal level, whereas it increased to only 180% in the hypoxic right ventricle. In the left ventricle, phenylephrine stimulated Ins(1,4,5)P_3 production to 160-170% in both normoxic and

hypoxic fetal hearts. In all the groups tested, prazosin completely abolished phenylephrine-stimulated Ins(1,4,5)P_3 production.

DISCUSSION

The major findings in the present study are: (1) long-term high-altitude hypoxemia significantly decreased α_1-adrenergic receptor density in the fetal right ventricle, but not the left, (2) long-term high-altitude hypoxemia decreased phenylephrine stimulated Ins(1,4,5)P_3 production in the fetal right ventricle, (3) a temporal relationships between Ins(1,4,5)P_3 and force existed in the hypoxic left ventricle, and (4) phenylephrine-stimulated production of Ins(1,4,5)P_3 was entirely α_1-adrenergic receptor mediated.

Previous in vivo studies in our laboratory have shown that long-term high-altitude hypoxemia significantly depressed fetal right ventricular function, with only slightly decreased left ventricular function, resulting in a reduction in cardiac output (10,11). Right ventricular sensitivity to afterload was also decreased. These studies showed that right ventricular function is more susceptible to long-term hypoxic effects than that of left ventricle, which was consistent with our present study. We demonstrated a significantly decreased α_1-adrenergic receptor density (Bmax) and Ins(1,4,5)P_3 response to agonist stimulation in the right ventricle of hypoxemic fetuses (Table1, Figure 6). Our finding also demonstrated that the normoxic right ventricle had the highest Ins(1,4,5)P_3 production among all the groups (Figure 6). It has been well established that α_1-adrenergic receptors mediate positive inotropism in the myocardium in addition to the well established role for β-adrenergic receptors (22,23,25). Thus, it is possible that the reduction in α_1-adrenergic receptors and Ins(1,4,5)P_3 production in the hypoxic right ventricle could contribute to the reduction in right ventricular output observed previously.

Our time-course study of force and Ins(1,4,5)P_3 production (Figure 2) compared favorably with that of Scholz et al (24). In their study with isolated electrically stimulated perfused rat hearts, Ins(1,4,5)P_3 increased significantly within 1 min. after stimulation, while force of contraction did not increase until 2 min. later. Thus both studies demonstrate that the increase in Ins(1,4,5)P_3 precedes the increase in force of contraction. Our finding provided further evidence that the α_1-adrenoceptor-mediated increase in Ins(1,4,5)P_3 and force of contraction were causally and temporal related. However, as shown in Figure 2, the content of Ins(1,4,5)P_3 returned to the basal level after about 5 min. and started to increase again at 15 min., while the agonist-induced contraction increased continuously and reached maximum at about 10 - 15 min.. Others have suggested that Ins(1,4,5)P_3-mediated release of Ca^{++} from sarcoplasmic reticulum works in initiating contraction, the sustained positive inotropic effect of phenylephrine may be caused by extracellular Ca^{++} influx (17,20,21).

It is generally accepted that phenylephrine stimulates α_1-adrenergic receptors in the heart muscle, which in turn activate phospholipase C. This phosphoinositide-specific enzyme mediates PtdIns(4,5)P_2 hydrolysis to generate Ins(1,4,5)P_3 and diacylglycerol (DAG) (2,6). Ins(1,4,5)P_3 releases calcium from intracellular stores such as sarcoplasmic reticulum, to enhance contraction. It has been shown that α_1-adrenergic receptor stimulation results in the formation of inositol phosphate in a concentration-dependent manner similar to that of the inotropic response (20). This was also evidenced in our present study in that there were phenylephrine concentration-dependent increases in both Ins(1,4,5)P_3 (Figure 4) and force generation (Figure 5). Thus, our study demonstrates a close correlation between phosphoinositide breakdown and inotropic responses mediated by α_1-adrenergic receptors in the fetal myocardium.

It has been shown that both $alpha_{1A}$ and $alpha_{1B}$ adrenergic receptor subtypes play a role in production of the positive inotropic effect mediated by myocardial α_1-adrenergic receptors and $Ins(1,4,5)P_3$ (8,18,27). Steinberg et al (26) showed that $Ins(1,4,5)P_3$ response to norepinephrine under hypoxic conditions was inhibited by prazosin and the $alpha_{1A}$ antagonist WB-4101 but not by $alpha_{1B}$ antagonist chloroethylclonidine. It has also been shown that both α_1-adrenergic receptor subtypes mediating norepinephrine-induced chronotropy were decreased significantly by hypoxia (13). We did not differentiate the effect of high-altitude hypoxemia on $alpha_{1A}$ and $alpha_{1B}$ subtypes in the present study. Further study is needed to address this issue.

In conclusion, we found that long-term high-altitude hypoxemia depressed myocardial α_1-adrenergic receptor density (Bmax) in the fetal right ventricle, but did not alter the dissociation constant (Kd). $Ins(1,4,5)P_3$ production in response to phenylephrine-stimulated was decreased in the fetal right ventricle following long-term high-altitude hypoxemia. We attribute the inhibition in $Ins(1,4,5)P_3$ production to the reduction in the α_1-adrenergic receptor number in the fetal right ventricle. We speculate that the decrease in α_1-adrenergic receptor density and $Ins(1,4,5)P_3$ in the fetal right ventricle during long-term hypoxia may be involved in the reduction in right ventricular function during long-term hypoxemic exposure.

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FIGURE LEGENDS

Figure 1. [^{3}H-Prazosin] saturation binding curves in fetal myocardium. Receptor density (Bmax) and dissociation constant (Kd) were calculated from each binding curve for each experiment and averaged for each group. Panel A: left ventricle (LV), Panel B: right ventricle (RV). Control (CON), Hypoxia (HYP). n=6-8.

Figure 2. Ins(1,4,5)P_3 (IP_3) production and force of contraction during 100 μM phenylephrine stimulation in isolated electrically driven papillary muscle strips from hypoxic fetal left ventricles (n=6).

Figure 3. Time-course of Ins(1,4,5)P_3 (IP_3) production during 100 μM phenylephrine stimulation in resting papillary muscle strips from normoxic fetal left ventricles (n=4).

Figure 4. Dose-response of Ins(1,4,5)P_3 (IP_3) to phenylephrine stimulation in resting papillary muscle strips from normoxic fetal right ventricles (n=4).

Figure 5. Phenylephrine dose-response in isolated electrically driven papillary muscle strips from hypoxic fetal left ventricles (n=6).

Figure 6. Phenylephrine-stimulated Ins(1,4,5)P_3 (IP_3) response in left ventricle (LV) and right ventricle (RV) (n=5-6) in both hypoxic and control fetuses. * significantly different from control left ventricle and hypoxic right ventricle. Phenylephrine (Phe), Prazosin(Praz).

Table 1. Myocardial α_1-adrenergic receptor density (Bmax) and dissociation constant (Kd) in fetal ovine membrane fractions.

		Bmax (fmol/mg)	Kd (nM)
LV	CON	22.32±2.58	0.26±0.05
	HYP	17.22±1.16	0.22±0.06
RV	CON	29.61±3.42	0.42±0.13
	HYP	18.73±2.05*	0.22±0.05

Values are mean ± SE, n = 6-8. Left Ventricle (LV), Right Ventricle (RV), Control (CON), Hypoxic (HYP). * - significantly different from control value.

Table 2. Phenylephrine-stimulated Ins(1,4,5)P_3 (IP_3) response in left (LV) and right (RV) ventricle from both hypoxic and control fetuses.

		PHE (pmol/mg protein)	PRAZ+PHE (pmol/mg protein)
LV	CON	17.13±1.20	7.72±0.84
	HYP	15.85±1.48	8.99±0.62
RV	CON	34.99±3.13*	12.74±1.47
	HYP	20.09±1.28	9.03±0.72

Values are mean ± SE, n = 5-6. Left Ventricle (LV), Right Ventricle (RV), Control (CON), Hypoxic (HYP), Phenylephrine (PHE), Prazocin (PRAZ). * - significantly different from control LV and hypoxic RV ($p<0.05$).

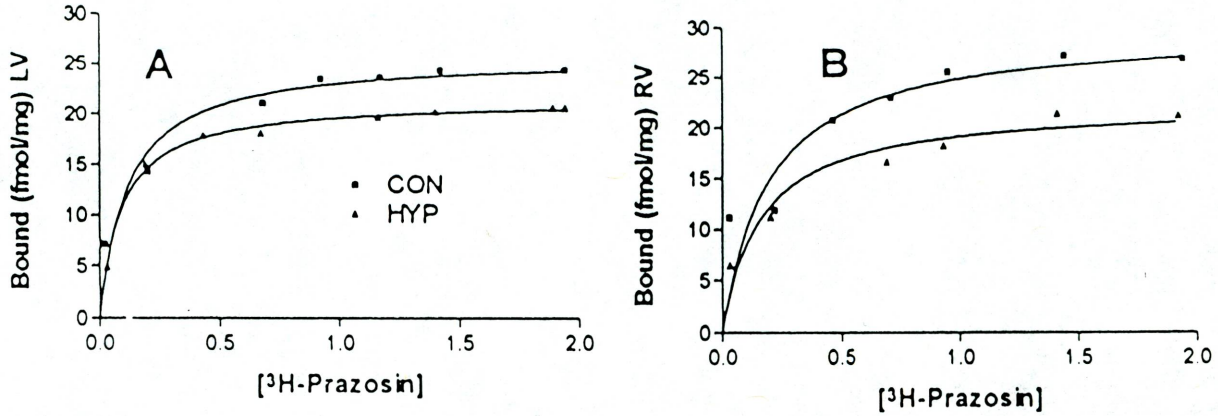

Figure 1. [^{3}H-Prazocin] saturation binding curves in control (CON) and hypoxic (HYP) fetal myocardium. Panel A: left ventricle (LV); Panel B: right ventricle (RV).

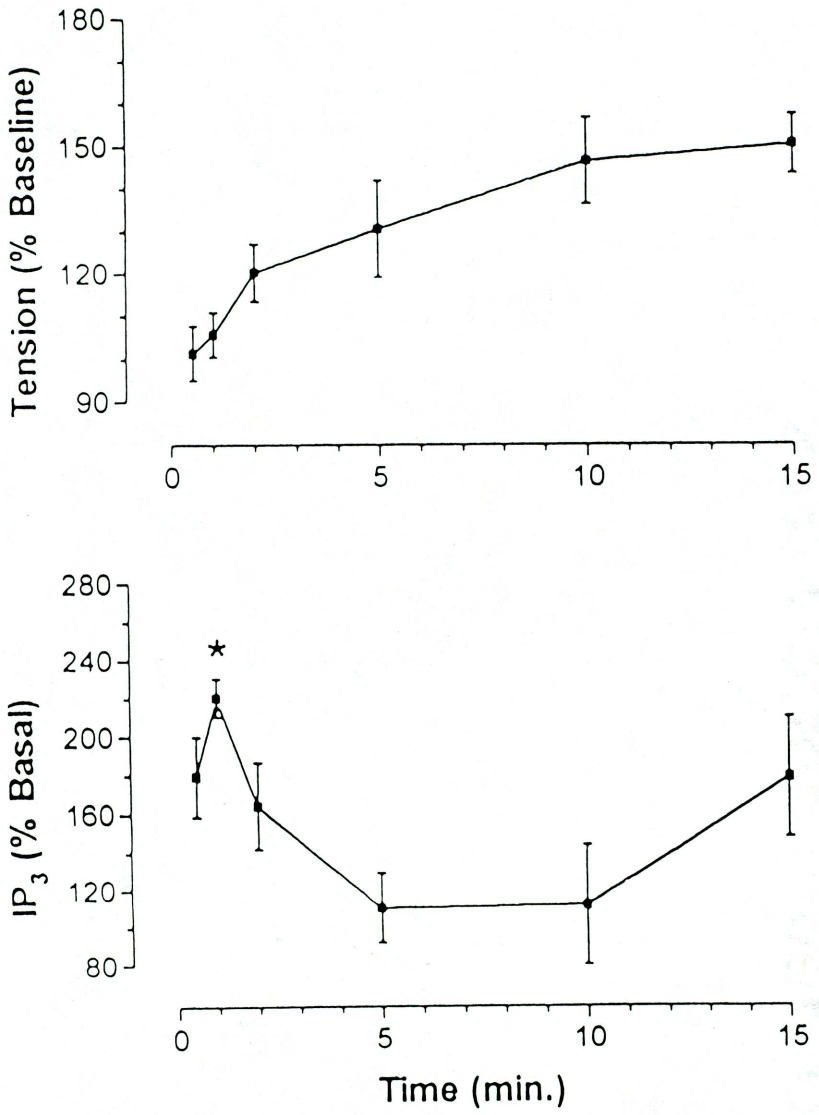

Figure 2. Ins(1,4,5)P_3 (IP_3) production and force of contraction during 100 μM phenylephrine stimulation in isolated electrically driven papillary muscle strips from hypoxic fetal left ventricles (n = 6).

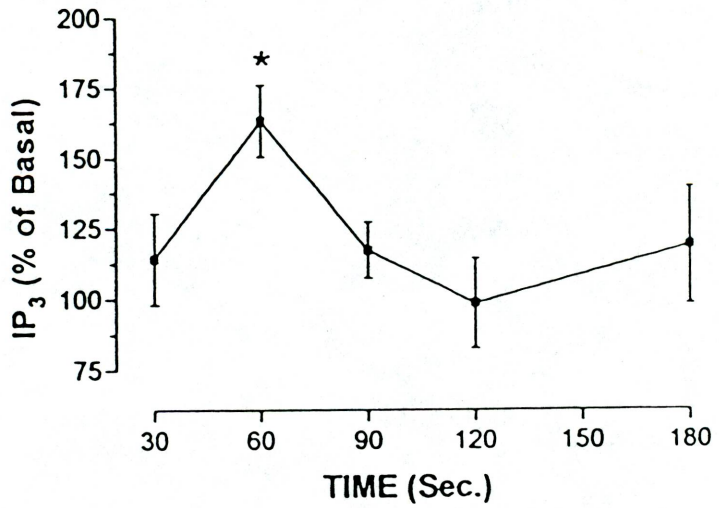

Figure 3. Time-course of Ins(1,4,5)P_3 (IP_3) production during 100 μM phenylephrine stimulation in resting papillary muscle strips from normoxic fetal left ventricles (n = 4).

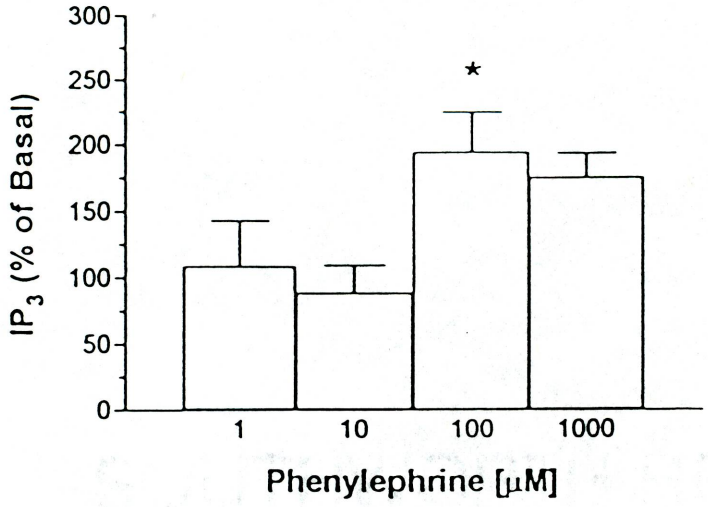

Figure 4. Dose-response of Ins(1,4,5)P_3 (IP_3) to phenylephrine stimulation in resting papillary muscle strips from normoxic fetal right ventricles (n = 4).

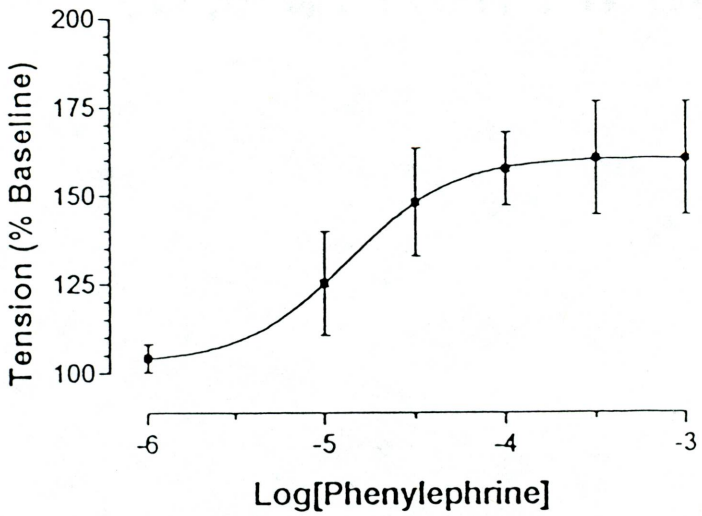

Figure 5. Phenylephrine dose-response in isolated electrically driven papillary muscle strips from hypoxic fetal left ventricles (n = 6).

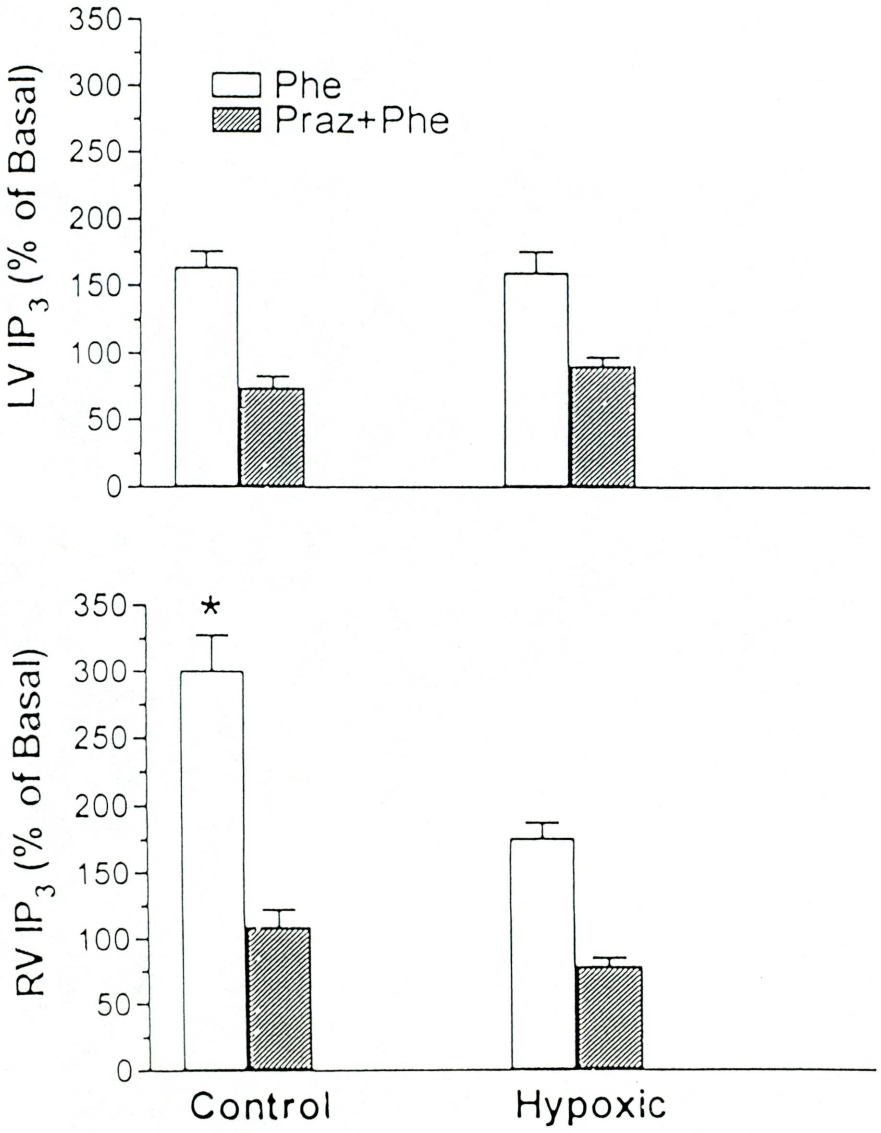

Figure 6. Phenylephrine-stimulated Ins(1,4,5)P_3 (IP_3) response in left (LV) and right (RV) ventricle from both hypoxic and control fetuses. * - significantly different from control LV and hypoxic RV. Phenylephrine (PHE), Prazocin (PRAZ). (n = 5-6).

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CHAPTER 5
CONCLUSION

General

Although chronic hypoxia causes increased perinatal morbidity and mortality, growth retardation, and low birth weight, the mechanisms underlying these sequelae are unclear (Jacobs R. et al., 1988). Previous *in vivo* studies indicate that long-term high-altitude hypoxemia significantly depresses fetal right ventricular function, but only slightly decreases left ventricular function, resulting in a reduction in cardiac output (Kamitomo M. et al., 1992) suggesting that hypoxia-induced changes in right ventricular contractility may contribute to altitude related increases in neonatal mortality. In the study by Kamitomo et al., the duration and intensity of hypoxia was similar to that used in the present studies; from 30 - 120 days gestation at an altitude of 3,820 m. In this key study, hypoxia produced no significant differences in left ventricular function as indicated by ventricular output or responses to increased preload. In contrast, hypoxia significantly depressed the relation between preload and ventricular output in the right ventricle. In addition, the slope of the relation between ventricular output and arterial pressure was also depressed by hypoxia, but only in the right ventricle. These data suggest that hypoxia preferentially and selectively depressed ventricular function in the right heart. The mechanisms responsible for this selective effect, however, remain unclear.

Hypoxia could potentially depress right ventricular function by depressing any of the factors which together determine ventricular output. These include heart rate and stroke volume, and also preload and afterload which directly influence stroke volume. The final factor governing ventricular output is myocardial contractility. As demonstrated by Kamitomo M et al. (1992), chronic hypoxia has no effect on heart rate

or preload. Although hypoxia has been shown to increase afterload, such increases can not completely explain the corresponding significant reduction in right ventricular output (approximately 35% reduction). For example, in the study by Kamitomo, hypoxia elevated fetal arterial pressure by only Å17%, but depressed right ventricular output by Å34%, suggesting that factors other than afterload are involved. Together with the absence of hypoxic changes in heart rate or preload, these data suggest that hypoxia depresses right ventricular contractility.

One important modulator of myocardium appears to be the myocardial adrenergic system, whose presence has been confirmed in many species and has been proposed to modulate cardiac rhythm and conduction as well as force of contraction (Endoh, M. et al., 1989). In terms of inotropic effects, α_1- and β- adrenergic systems appear to work together such that the α_1 component contributes 25% and the β- adrenergic receptor component accounts for 75% of the final inotropic effect of norepinephrine in the isolated rat heart (Skomedal et al., 1988). In addition, α_1-adrenergic receptors also appear involved in changes in gene expression which governs myocardial cell growth (Terzic et al., 1993).

Given the capacity of the myocardial adrenergic system to modulate myocardial contractility, it is possible that hypoxia could effect changes in contractility by altering adrenergic function in the heart. To examine this possibility, a preliminary dose-response study to methoxamine (an α_1-receptor agonist) was performed using right and left ventricular papillary muscles from normoxia and hypoxic fetuses (Kamitomo M et al., unpublished data). Hypoxia had no effects on responses to methoxamine in the left heart but severely attenuated reactivity to methoxamine in the right heart, and thus reinforced the view that the observed hypoxic depression of right ventricular function in the fetal heart could be mediated via selective modulation of the adrenergic system. We therefore

proposed to further study the myocardial α_1-adrenergic receptor system by more completely characterizing α_1-receptor dose-response relations, by measuring α_1-receptor density, by quantifying changes in inositol triphosphate {Ins(1,4,5)P_3} responses following α_1-receptor agonist stimulation. We also decided to test the possibility that changes in adrenergic function in hypoxic hearts involves altered interactions between the α_1 and β_1- adrenergic receptor systems by examining interactive effects of α_1 and β_1 on dose-response relations. Together, these studies enable a direct assessment of the hypothesis that hypoxia depresses right ventricular function in the ovine fetus by selectively depressing adrenergic function.

Conclusions and Discussion

First of all, our studies demonstrated that the α_1-adrenergic receptor agonist, phenylephrine, exerted a positive inotropic effect in the fetal heart. Other investigators have previously demonstrated similar findings in newborns and adult animals (Nakanishi, T. et al., 1989). Our study was the first to confirm the finding in the fetuses. Long-term high-altitude hypoxemia 1) depressed myocardial α_1-adrenergic receptor density (Bmax) in the fetal right ventricle, but did not alter the dissociation constant (Kd). It is generally accepted that α_1-adrenergic stimulation of myocardial α_1- receptors causes hydrolysis of phosphotidylinositol biphosphate to form Ins(1,4,5)P_3, which then activates calcium release from intracellular stores (Berridge M. J. et al., 1993). 2) Ins(1,4,5)P_3 production in response to phenylephrine-stimulation was also decreased in the fetal right ventricle following long-term high-altitude hypoxemia. 3) The ratio between the IP_3 response and the receptor density changed between the Con and Hyp. The ratio between the IP3 response and the receptor density for LV were: 17.13/22.32=0.77 for Con, and 15.85/17.22=0.92 for Hyp. The ratio between the IP3 response and the receptor density

for RV were: 34.99/29.61=1.18 for Con, and 20.09/18.73=1.07 for Hyp. This ratio is changed between the Con and Hyp groups in both LV and RV. It demonstrates that hypoxia has multiple effects: 1) it differentially depresses Bmax in right heart only, and 2) it also non-specifically depresses IP3/Bmax ratio (coupling efficiency). Therefore, both the changes in density and in coupling are probably involved in the decreased IP3 response. In addition, α_1-adrenergic receptors mediate positive inotropism in the myocardium in addition to the well established role for β_1- adrenergic receptor systems (Skomedal M. et al., 1988). The α_1-adrenergic receptor system plays a significant role in contractility under normal condition. Therefore, during long-term hypoxia, we speculate that the decrease in α_1-adrenergic receptor density and Ins(1,4,5)P_3 in the fetal right ventricle may be involved in the reduction in right ventricular response. A decreased right ventricular response could in turn explain why cardiac output is reduced following hypoxia.

Secondly, our findings showed that hypoxemia augmented the dose-response to the β-adrenergic agonist isoproterenol in isometrically-contracted papillary muscle preparations from the left ventricle. Studies done in our laboratory have also demonstrated that there is a 55% increase in right ventricular β-adrenergic receptor density in hypoxic fetuses, but no change in the left ventricle. However, Both isoproterenol-stimulated and forskolin-stimulated cAMP levels were 1.4 to 2-fold higher than the controls in both hypoxic ventricles(Browne V. et al. in press). The up-regulating effect on β- adrenergic receptor density and cAMP level in the hypoxic right ventricle has been used to explain the relatively higher sensitivity of this ventricle to afterload changes, *i.e.* the elevated arterial pressure in the hypoxic condition (Browne V. et al. in press). Although there was no change in the left ventricle in regard to β- adrenergic receptor number, cAMP was obviously elevated in that ventricle. These results strongly suggest

that hypoxia acts downstream of second-messenger production in the signal transduction cascade coupled to β- adrenergic receptor system. Therefore, cAMP-mediated events such as activation of protein kinase A and phosphorylation of calcium channels, troponin and phospholamban are expected to be enhanced (Zong, X, et al, 1995). We observed in the present study, in that hypoxia augmented isoproterenol dose-response in the left ventricle. As mentioned above, myocardial α_1- adrenergic receptor function was depressed by hypoxia, especially in the right ventricle.

Thirdly, our studies showed that α_1- adrenergic receptor activation exerted an antagonizing effect on β_1- adrenergic receptor system in the fetal left ventricle following long-term high-altitude hypoxemia. This is also compatible with previous literature (Buxton I. L. et al., 1986). It has been shown that incubation of intact cardiac myocytes with norepinephrine leads to significantly less cyclic AMP accumulation than stimulation with either norepinephrine plus prazosin or isoproterenol, due to activation of cAMP phosphodiesterase (Buxton I. L. et al., 1986). Electrophysiological studies have indicated that myofibrillar responsiveness to Ca^{2+} is increased with phenylephrine, but decreased with isoproterenol (Endoh M. et al., 1988). Our study only demonstrated the receptor interaction at the functional level. However, adaptive changes to chronic hypoxia may have occurred at the level of the receptor, second-messenger mediators such as $Ins(1,4,5)P_3$, protein kinase C (PKC), cAMP and downstream mediators such as protein kinase A (PKA) and effector proteins in the contractile apparatus in the myocardium. Further study is needed to clarify this issue. Our findings regarding adrenergic function provided new insight into understanding the decreased cardiac output observed in hypoxic fetuses. Apparently, the myocardial α_1- adrenergic receptor system was significantly changed by hypoxia . The down-regulation of the α_1- adrenergic receptor

system in the hypoxic right ventricle could be responsible for reduced right ventricular output.

Finally, our studies showed that myocardial α_1-adrenergic receptor density decreased during development from the fetus to the adult. The dissociation constants in the fetus were also different from those in the adult, indicating that myocardial α_1-adrenergic receptor subtypes may have been changed with development. For a receptor subtype, its properties will be conserved in different tissues. We can assume that receptor-binding studies are done under similar conditions, i. e. with the same local ionic charges and the same charge distribution. Thus, the pharmacological profile of the receptor subtype (e.g. K_D for a particular antagonist) should be the same. regardless of whether it is derived for tissues containing a single subtype or tissues containing multiple subtypes. Based on this concept, the difference in K_D values can point to the existing of different receptor subtypes. However, the inotropic response to phenylephrine stimulation was not significantly altered during development. This may be due to changes in coupling efficiency of receptors. It may also be caused by existence of spare receptors in the myocardium of fetuses or adults. Our findings agree with previous literature (Kimball K.A. et al., 1991). It has been shown in rat cultured myocytes that the level of α_1-adrenergic receptor mRNA, as determined by Northern blot analysis, decreased with age (Kimball K.A. et al., 1991). Studies have shown that cultured myocytes can accurately reflect changes in intact muscle (Hryshko, L.V., et al. 1989). In addition, sarcoplasmic reticulum (SR) is underdeveloped in the premature heart (Nakanishi T. et al., 1984). Currently, three mechanisms have been proposed to participate in the positive inotropic effect of α_1-adrenoceptor agonists: (a) an indirect increase in Ica inward current, (b) a stimulation of inositol phosphate turnover, and (c) an increases in myofibrillar responsiveness to Ca^{2+} (Terzic, A., M. 1993). Release of SR

Calcium is involved in the regulation of contractility. Therefore, it is possible that the amount of calcium stored in SR in the premature heart is less, resulting in a reduced response per number of α_1-adrenergic receptors stimulated. This could at least partially explain why adult hearts contract as vigorously as fetal hearts in response to α_1-receptor stimulation although there are fewer α_1-receptors in the adults. We speculate that changes in the coupling process subsequent to receptor stimulation, together with alterations in signal transduction pathways, may be crucial in explaining changes that occur in the myocardial α_1-adrenergic receptor system during development.

As shown in Figure 1, stimulation of myocardial α_1- adrenergic receptors may increase the sensitivity of the contractile proteins for Ca^{2+} (Pathway 1). This is probably related to activation of Na^+/H^+ exchange and alkalization of the sarcoplasm, which leads to increased Na^+/Ca^{2+} exchange, resulting in increase in $[Ca^{2+}]_i$. The α_1- adrenoceptor stimulation causes phosphorylation of contractile protein(s) and alkalization by activation of the Na/H antiport. This in turn causes myofilament Ca-sensitization, which has been a proposed mechanism underlying the positive inotropic action of α_1- adrenergic receptors. The rapid and transient increase in $[Ca^{2+}]_i$ that occurs prior to the generation of force plays a central role in excitation-contraction coupling in intact cardiac muscle.

In addition, α_1- adrenergic receptor stimulation activates phospholipase C. The stimulation of cardiac α_1- adrenergic receptors promotes the breakdown of PI, producing IP3 and DAG. IP3 as a second-messenger in the α_1- adrenergic receptor mediated inotropic action has been demonstrated by Scholz et al. (1992). They found that the positive inotropic effect of phenylephrine in rat atria is preceded by a decrease in PIP_2 and an increase in IP3. Otani et al. (1988) exposed papillary muscles labeled with [^{3}H]inositol to 0.1 mM neomycin, a blocker of PIP_2 degradation. Neomycin inhibited [^{3}H] inositol phosphate formation and diminished the inotropic effects normally induced

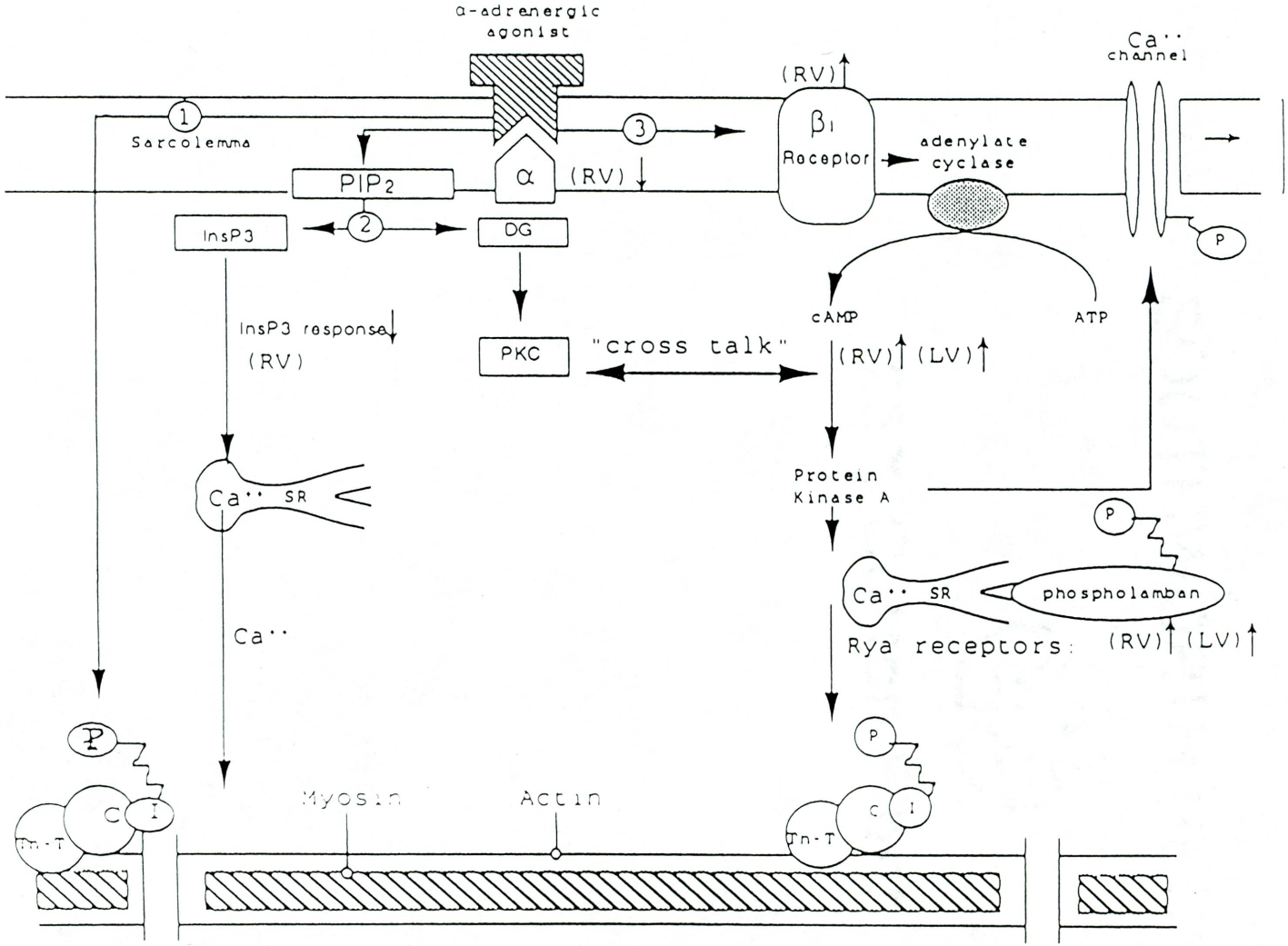

Figure 1. Effect of long-term high altitude hypoxia on receptor function and second-messenger systems in the heart.

by α_1-adrenergic receptor agonist. Their study shows that the hydrolysis of PIP_2 and Phopholipase C activation is an essential link in the pharmacomechanical coupling that follows the binding of the agonist to cardiac α_1-adrenergic receptors. This phosphoinositide-specific enzyme mediates ptdIns(4,5)P_2 hydrolysis to generate Ins(1,4,5)P_3 and diacylglycerol (DAG) (Pathway 2). Ins(1,4,5)P_3 releases calcium from intracellular stores, such as sarcoplasmic reticulum (SR), to enhance contraction. α_1-adrenergic receptor stimulation in the heart also leads to changes in membrane potential (prolongation of the action potential), phospholamban phosphorylation state, and even myofilament calcium sensitization (Terzic, A., et al. 1993). Studies have shown that Ins(1,4,5)P_3 receptors could be converted from low-affinity to high-affinity binding sites as a consequence of decreased SR Ca^{2+} content (Sugiyama T. et al., 1995). Our current study has shown that Ins(1,4,5)P_3 response to α_1-adrenergic receptor stimulation is decreased in the right ventricle in hypoxic fetuses (hypoxic RV 176.58 ± 11.31 vs. normoxic RV 300.90 ± 26.95, pmol/mg protein). We speculate that the reduction in Ins(1,4,5)P_3 response could also be due to a decrease in Ins(1,4,5)P_3 receptor density and activity. Thus, SR Ca^{2+} release is decreased in the hypoxic right ventricle, leading to decreased myocardial contractility.

Speculations and Future Experiments

PKC is also activated in response to myocardial α_1-adrenergic receptor stimulation. PKC participates in the "cross talk" between cAMP and the polyphosphoinositide signaling cascade. Samson R.A. et al. (1994) has shown that PKC participates in the "cross talk" between cAMP and the phosphoinositide signaling pathways. Exposure to the PKC-activating phorbol esters results in suppression of the β_1-adrenergic-induced triggered activity in hypertrophic cardiomyopathic preparations

(Samson R.A. et al., 1994) (Samson R.A. et al., 1994). Our study has shown that activation of α_1-adrenergic receptors antagonizes the effect of β_1- adrenergic receptors in the hypoxic left ventricle. We speculate that activation of PKC in response to α_1-adrenergic receptor stimulation depresses cAMP levels in the left ventricle following hypoxia. In addition, various "cross-talk" regulations in the phosphoinositide-cAMP signal transduction pathways such as PKC and PKA, Ins(1,4,5)P_3 and cAMP could also be modified by hypoxia (Di Marzo, V. et al., 1991).

Hypoxia stimulates gene expression in many organisms. For example, the erythropoietin gene for red cell production is turned on in response to hypoxia (Baker R. 1984). It has been proposed that oxygen could interact directly with nuclear proteins to induce binding, or that it could modify proteins bound to DNA to induce transcription of the erythropoietin gene (Ratcliffe P. et al., 1995). Some genes are transcribed in an oxygen-dependent manner such as platelet-derived growth factor β chain and hypoxically inducible nuclear factor HIF-1 (Ractcliffe P. et al., 1995). It is reasonable to infer that an oxygen-sensing system similar to the one involved in erythropoietin regulation exists in the heart, and that it modifies the myocardial adrenergic receptor system during hypoxia.

In summary, our findings regarding adrenergic function provided new insight into understanding the decreased cardiac output observed in hypoxic fetuses. Apparently, the myocardial α_1-adrenergic receptor system plays an important role in the hypoxic condition. The down-regulation of the α_1-adrenergic receptor system in the hypoxic right ventricle was responsible for reduced right ventricular output. We speculate that hypoxia modifies various receptors, second-messenger mediators and "cross-talk" between the phosphoinositide-cAMP cascades.

Additional studies are needed to investigate the effects of hypoxia and development upon adrenergic receptor systems. Levels of α_1-adrenergic receptor mRNA

can be determined by Northern blot analysis. In addition, α_1-adrenergic receptor subtypes such as α_{1A} and α_{1B} can be identified by dose-response studies to an α_1-adrenergic receptor agonist, by radioligand binding study, and by Ins(1,4,5)P_3 studies in the presence of pharmacologically specific antagonists such as WB4101 and CEC. In regard to receptor interaction and cross-talk regulation, cAMP levels can be measured in response to myocardial α_1-adrenergic receptor stimulation or Ins(1,4,5)P_3 and PKC responses can be detected following stimulation of cAMP production by isoproterenol and forskolin. These studies should further elucidate the changes in the cardiovascular system that occur during hypoxia and development.

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