6-1996

Myocardial Contractility in Fetal Sheep Exposed to Long-term Hypoxia at High Altitude : Activator Calcium and Beta-adrenergic Receptor Function

Vaughn A. Browne

ABSTRACT

MYOCARDIAL CONTRACTILITY IN FETAL SHEEP EXPOSED TO LONG-TERM HYPOXIA AT HIGH ALTITUDE: ACTIVATOR CALCIUM AND BETA-ADRENERGIC RECEPTOR FUNCTION

by

Vaughn A. Browne

We studied myocardial contractility in fetal sheep exposed to ~112 days of hypoxia at high-altitude (3,820 m). We recorded left and right ventricular wet weight, and measured the inotropic responses to extracellular calcium $[Ca^{2+}]_o$ (0.2-10 mM), ryanodine (10^{-10}–10^{-4} M), isoproterenol (10^{-10}–10^{-6} M), and forskolin (0.01–10 μM) in isometrically contracting papillary muscles. In addition, we quantified dihydropyridine (DHPR), ryanodine (RyR), and β-adrenergic receptor densities, and measured basal and stimulated intracellular cAMP levels. In hypoxic fetuses, left ventricular wet weight was unchanged, but right ventricular weight was ~20% lower than controls. Curves describing the force-$[Ca^{2+}]_o$ relationship were left-shifted, and the top plateaus were decreased by ~35% in both left and right ventricles. Ryanodine (10^{-4} M) reduced maximum active tension (T_{max}) to ~25–40% of baseline values, indicating that the sarcoplasmic reticulum was the chief source of activator calcium. DHPR number did not change, but RyR density and the RyR:DHPR ratios in both ventricles were higher in hypoxic fetuses. At the highest concentration of isoproterenol (10 μM), maximum active tension was ~32% and ~20% lower than controls in hypoxic left and right ventricles, respectively. The contractile response to forskolin was severely attenuated in both hypoxic ventricles. β-receptor

density was unchanged in the left ventricle, but increased by 55% in the hypoxic right ventricle. K_D was not different from controls in either ventricle. Basal cAMP levels were not different from controls, but isoproterenol-stimulated and forskolin-stimulated cAMP levels were 1.4 to 2-fold higher than controls in both hypoxic ventricles.

In summary, there was no ventricular hypertrophy, and hypoxia decreased contractility, possibly by reducing the availability of activator calcium. The blunted contractile responses to isoproterenol and forskolin were not related to down-regulation of the β-adrenergic receptors or adenylate cyclase. We speculate that the changes in the inotropic responsiveness to both calcium and β-agonists are linked by a common, as yet unexplored, mechanism, possibly involving decreased A-kinase activity or increased phosphatase activity. The expected changes in the phosphorylation state of several key effector proteins, that would, theoretically, occur in that scenario, are consistent with the observations in study.

Loma Linda University

Graduate School

Myocardial Contractility in Fetal Sheep Exposed to Long-term Hypoxia at High Altitude: Activator Calcium and Beta-Adrenergic Receptor Function

by

Vaughn A. Browne

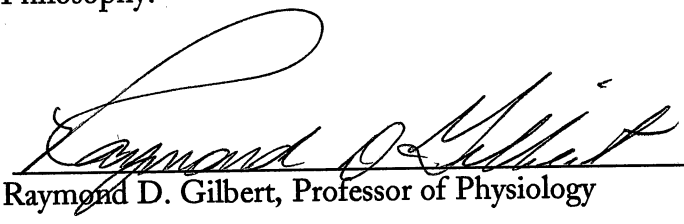
A Dissertation in Partial Fulfillment

of the requirements for the Degree

Doctor of Philosophy in Physiology

June 1996

Each person whose signature appears below certifies that this dissertation in their opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.



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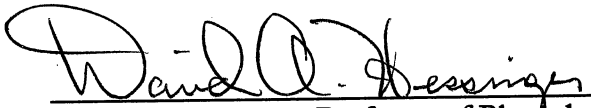

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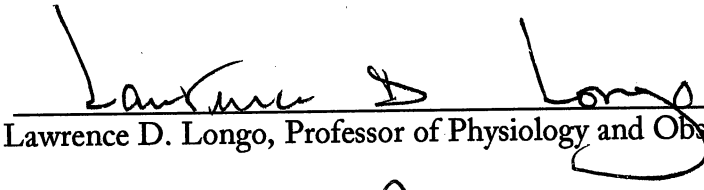

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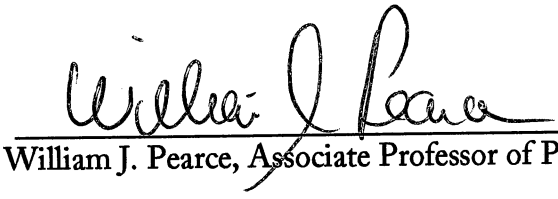

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ACKNOWLEDGMENTS

This project would not have been possible without the inspiration, guidance, and support provided by several key individuals. With deep appreciation, I thank Dr. David Taylor and Mrs. Maxine Taylor, who made it possible for me to study at Pacific Union College, where I received an excellent undergraduate education. Drs. William Hemmerlin, Terrence Trivett, Isaac Johnson, and Sherman Nagel guided my early development, and encouraged me to pursue a life of scholarship and service to humanity. It was their selfless dedication to students, their high-academic standards, and passion for discovery that inspired me to a career in academic medicine.

Drs. David Baylink, David Hessinger, Lawrence Longo, Barton Rippon, Barry Taylor, Anthony Zuccarelli, and the late Robert Boucek introduced me to the Medical Scientist Training Program. Their continued support was invaluable in the completion of this project. I am deeply indebted to Drs. Raymond Gilbert, Donald Bers, Allan Brady, David Hessinger, Lawrence Longo, and William Pearce, who guided my project, created an environment that encouraged independent scientific inquiry, provided key scientific insights and methodology, and modeled excellence. Their patience and unyielding financial and moral support were critical to the completion of this project.

I thank Drs. James Anholm, Charles Houston, Lorna Moore, John Sutton, Peter Wagner, and John West, who excited my interest in hypoxia and high-altitude physiology. I appreciate Dr. Grenith Zimmerman, whose guidance in statistical analysis and experimental design were invaluable. Virginia Stiffel and Al VanVarick provided valuable technical assistance.

Finally, I thank my parents, K. Arnaud and Floretta Browne, my wife, Lucille, my brothers and sisters and their families, and my closest friends who provided much needed encouragement, financial support, and emotional support.

This work was supported in part by USPHS grants: HD22190 and HD03807.

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CHAPTER ONE

INTRODUCTION

The mammalian fetus grows and develops in an environment characterized by low oxygen tension (P_{O_2}). Despite increases in maternal blood volume, cardiac output, and uterine blood flow during pregnancy, which optimize the delivery of oxygen to the fetus via the placenta, fetal arterial P_{O_2} equals 25–30 Torr (26), a value similar to that observed in climbers at the summit of Mt. Everest (46). Paradoxically, fetal oxygen consumption is ~2 times that of the adult (29), a consequence of higher metabolic rate. For example, in the ovine fetus, metabolic rate is ~35 $kcal \cdot min^{-1} \cdot kg^{-1}$ compared to ~20 $kcal \cdot min^{-1} \cdot kg^{-1}$ in resting nonpregnant adults (17). Several adaptations allow the mammalian fetus to sustain organ growth and differentiation in the face of its physiologic hypoxia. Fetal hemoglobin has a higher affinity for oxygen than maternal hemoglobin (7, 16, 26), and hematocrit is higher in fetuses, so that the total oxygen carrying capacity is 10%–20% greater than in the adult (26). However, fetal oxygen reserve is only enough to meet its high metabolic demands for 1–2 min. To compensate, fetal cardiac output per milliliter of oxygen consumed is approximately 2.5 times higher than in the adult, so that fetal tissues are perfused at a higher rate (18, 41). Umbilical blood flow and placental oxygen exchange are exquisitely dependent on fetal cardiac output and its distribution (26). Thus, high cardiac output must be maintained to prevent fetal oxygen tensions from falling below critical levels.

The regulation of fetal cardiac performance and blood flow distribution has been the subject of intense study over the past twenty five years. The four determinants of cardiac

performance, heart rate, preload, afterload and contractility have been well characterized in the fetal lamb. Several investigators have shown that high heart rate (5, 6, 37, 36), which tends to be the same among mammals of different species (33), is critical in maintaining fetal cardiac output and adequate oxygen delivery. Other investigators (14, 15, 43, 44) have shown that when stroke volume is related to mean atrial filling pressure, the fetal heart operates near the plateau of its ventricular function curve, thus appearing to have little reserve for increasing its output. When stroke volume was related to fetal arterial pressure, the right ventricle was more adversely affected by increases in arterial pressure than the left ventricle (43, 44). Several studies in chronically instrumented fetal lambs in utero (4, 42), and in isolated muscle (2, 4, 32) have demonstrated that contractility increases with fetal age, particularly during the last week before birth and the first week of neonatal life (4).

Since the first measurements of fetal cardiac output *in utero* (38), investigators have had particular interest in how fetal cardiac performance adapts to the stress of maternal hypoxia, maternal arterial oxygen tension being a key determinant of placental oxygen exchange (13, 26, 27). In response to acute hypoxia, fetal heart rate decreases (12, 28, 45), arterial pressure increases (12, 28, 45), and blood flow is redistributed to the brain, heart, and adrenals at the expense of blood flow to other organs (12, 28, 35, 40). In addition, plasma concentrations of several hormones increase, including catecholamines, vasopressin , renin, and erythropoietin (11, 24, 30, 39), and ACTH and cortisol (9, 10). When hypoxia persists for up to 48 h, the redistribution of blood flow is maintained, although heart rate and arterial pressure return to normal values (8). Surprisingly, fetal

cardiac output is either unchanged or slightly decreased during acute hypoxia (12, 28, 35).

Until recently, comparatively little was known about how the fetus adapts to long-term hypoxia. Prolonged hypoxia is associated with increased perinatal morbidity and mortality (25, 31), growth retardation (31), and low birth weight (25, 34). Our lab evaluated the effects of long-term high-altitude hypoxia on ovine fetal right (RV) and left (LV) ventricular performance *in vivo* (1, 19, 20, 21, 22). In studies of fetal cardiac function during 2-weeks of hypoxia (1, 20), the RV and LV operated near the plateau of their function curves with little preload reserve in both normoxic and hypoxic fetuses. After 3 days of hypoxia, RV output decreased by 30% and remained decreased after 14 days. After 7 days of hypoxia, LV output decreased by 21% and remained decreased (~38%) after 14 days. Sensitivity to afterload decreased markedly in the RV after 14 days, but was unchanged in the LV. In another study (21), hypoxia was maintained during days 30–134 of gestation. Both fetal ventricles operated near the plateau of their function curves with little preload reserve in both normoxic and hypoxic fetuses. However, the plateau of the right ventricular function curve was significantly lower (~35%) in hypoxic fetuses. Left ventricular output was not significantly reduced (~85% of controls). Stroke volume was reduced by ~38% and ~20% in the hypoxic right and left ventricles, respectively. As seen previously, sensitivity to afterload was decreased in the right ventricle of hypoxic fetuses, but was unchanged in the left ventricle.

Hypoxia significantly increased systemic arterial pressure (afterload) in all studies (1, 19, 20, 21). However, the increased arterial pressures could only account for ~25% of the reduction in RV output. Heart weight did not change significantly and there was no

evidence of ventricular hypertrophy (21). Blood flow to the heart increased (19) so that oxygen delivery to the heart was maintained despite the decrease in arterial P_{O_2}. Because heart rate did not differ between normoxic and hypoxic fetuses (1, 20, 21, 22), it is unlikely that the reduction in cardiac performance was due to a reduction in diastolic filling time (3, 5), or to rate related changes in inotropy (force-frequency relationship) (2, 23). Furthermore, the similarity in heart rate suggests that the mechanisms that control fetal heart rate were not altered by long-term hypoxia. Recently, our lab demonstrated that the chronotropic response to isoproterenol was preserved in fetuses exposed to long-term hypoxia (22). However, the increase in cardiac output was smaller in the hypoxic group, indicating a decrease in the positive inotropic response to isoproterenol.

Because the reduction in cardiac performance observed in hypoxic fetuses could not be completely explained by changes in preload, afterload, or heart rate, we hypothesized that myocardial contractility may be decreased during long-term hypoxia. The purpose of this study was to investigate contractility in isolated cardiac muscle taken from normoxic and chronically hypoxic fetuses during the last week of fetal life. Contractility increases with fetal age, particularly during the last week before birth and the first week of neonatal life (4). We reasoned that if long-term hypoxia alters myocardial contractility, the changes would most likely be apparent during the last week of fetal life when inotropic state increases.

REFERENCES

1. Alonso, J. G., T. Okai, L. D. Longo, and R. D. Gilbert. Cardiac function during long-term hypoxemia in fetal sheep. *Am. J. Physiol.* 257: H581–H589, 1989.

2. Anderson, P. A. W., A. Manring, and C. Crenshaw. Biophysics of the developing heart I. The force-interval relationship. *Am. J. Obstet. Gynecol.* 138: 33–43, 1980.

3. Anderson, P. A. W., A. P. Killam, R. D. Mainwaring, and A. E. Oakeley. In utero right ventricular output in the fetal lamb: the effect of heart rate. *J. Physiol. (Lond.)* 387: 297–316, 1987.

4. Anderson, P. A. W., K. L. Glick, A. Manring, and C. Crenshaw Jr. Developmental changes in cardiac contractility in fetal and postnatal sheep: in vitro and in vivo. *Am. J. Physiol.* 247 (*Heart Circ. Physiol.* 16): H371–H379, 1984.

5. Anderson, P. A. W., K. L. Glick, A. P. Killam, and R. D. Mainwaring. The effect of heart rate on in utero left ventricular output in the fetal sheep. *J. Physiol. (Lond.)* 372: 557–573, 1986.

6. Anderson, P. A., A. P. Killan, R. D. Mainwaring, and A. E. Oakeley. In utero right ventricular output in the fetal lamb: the effect of heart rate. *J Physiol. Lond.* 387: 297–316, 1987.

7. Battaglia, F. C., W. Bowes, H. R. McGaughey, E. L. Makowski, and G. Meschia. The effect of fetal exchange transfusions with adult blood upon fetal oxygenation. *Pediatr. Res.* 3: 60–65, 1969.

8. Bocking, A. D., R. Gagnon, S. E. White, J. Homan, K. M. Miline, and B. S. Richardson. Circulatory responses to prolonged hypoxemia in fetal sheep. *Am. J. Obstet. Gynecol.* 159: 1418–1424, 1988.

9. Boddy, K., C. T. Jones, C. Mantell, J. G. Ratcliffe, and J. S. Robinson. Changes in plasma ACTH and corticosteroid of the maternal and fetal sheep during hypoxia. *Endocrinology* 94: 558–591, 1974.

10. Boshier, D. P., H. Holloway, and G. C. Liggins. Effects of ACTH and cortisol on adrenocortical growth and cytodifferentiation in the hypophysectomized fetal sheep. *J. Dev. Physiol.* 3: 355–373, 1981.

11. Cohen, W. R., G. J. Piasecki, and B. T. Jackson. Plasma catecholamines during hypoxemia in fetal lambs. *Am. J. Physiol.* 243 (*Regulatory Integrative Comp. Physiol.* 12): R520–R525, 1982.

12. COHN, H. E., E. J. SACKS, M. A. HEYMANN, AND A. M. RUDOLPH. Cardiovascular responses to hypoxemia and acidemia in fetal lambs. *Am J. Obstet. Gynecol.* 120: 817–824, 1974.

13. EDELSTONE, D. I., B. B. PETICCA, AND L. J. GOLDBLUM. Effects of maternal oxygen administration on fetal oxygenation during reductions in umbilical blood flow in fetal lambs. *Am. J. Obstet. Gynecol.* 152: 351–358, 1985.

14. GILBERT, R. D. Control of fetal cardiac output during changes in blood volume. *Am. J. Physiol.* 238: H80–H86, 1980.

15. GILBERT, R. D. Effects of afterload and baroreceptors on cardiac function in fetal lambs. *J. Develop. Physiol.* 4: 299–310, 1982.

16. HUGGETT, A. ST. G. Foetal blood-gas tensions and gas transfusion through the placenta of the goat. *J. Physiol. Lond.* 62:373–384, 1927.

17. JONES, C. T., AND T. P. ROLPH. Metabolism during fetal life: a functional assessment of metabolic development. *Physiol. Rev.* 65(2): 357–430, 1985.

18. JONES, M. D. JR., L. I. BURD, E. L. MAKOWSKI, G. MESCHIA, AND F. C. BATTAGLIA. Cerebral metabolism in sheep: A comparative study of the adult, the lamb and the fetus. *Am. J. Physiol.* 229: 235–239, 1975.

19. KAMITOMO, M., J. G. ALONSO, T. OKAI, L. D. LONGO, AND R. D. GILBERT. Effects of long-term, high-altitude hypoxemia on ovine fetal cardiac output and blood flow distribution. *Am. J. Obstet. Gynecol.* 169:701–707, 1993.

20. KAMITOMO, M., L. D. LONGO, AND R. D. GILBERT. Cardiac function in fetal sheep during two weeks of hypoxemia. *Am. J. Physiol.* 266 (*Regulatory Integrative Comp. Physiol.* 35): R1778–R1785, 1994.

21. KAMITOMO, M., L. D. LONGO, AND R. D. GILBERT. Right and left ventricular function in fetal sheep exposed to long-term high-altitude hypoxemia. *Am. J. Physiol.* 262: H399–H405, 1992.

22. KAMITOMO, M., T. OHTSUKA, AND R. D. GILBERT. Effects of isoproterenol on the cardiovascular system of fetal sheep exposed to long-term high-altitude hypoxemia. *J. Appl. Physiol.* 78(5): 1793–1799, 1995.

23. LEWARTOWSKI, B. AND B. PYTKOWSKI. Cellular mechanism of the relationship between myocardial force and frequency of contractions. *Prog. Biophys. Molec. Biol.* 50: 97–120, 1987.

24. LEWIS, A. B., W. N. EVANS, AND W. SISCHO. Plasma catecholamine responses to hypoxemia in fetal lambs. *Biol. Neonate* 41: 115–122, 1982.

25. LICHTY, J. A., R. Y. TING, P. D. BRUNS, AND E. DYAR. Studies of babies born at high altitude. I. Relation of altitude to birth weight. *Am. Med. Assoc. J. Dis. Child.* 93: 666–670, 1957.

26. LONGO, L. D. Respiratory gas exchange in the placenta. In: *Handbook of Physiology The Respiratory System, Gas Exchange.* Bethesda, MD: Am. Physiol. Soc. Section 3, vol. IV, chapt. 18, pp. 351–401, 1987.

27. LONGO, L. D., E. P. HILL, AND G. G. POWER. Theoretical analysis of factors affecting placental O_2 transfer. *Am. J. Physiol.* 222:730–739, 1972.

28. LONGO, L. D., J. F. WYATT, C. W. HEWITT, AND R. D. GILBERT. A comparison of circulatory responses to hypoxic hypoxia and carbon monoxide hypoxia in fetal blood flow and oxygenation. In: *Fetal and Newborn Cardiovascular Physiology*, edited by L. D. Longo and D. D. Reneau. New York: Garland, 1978, p 259–287.

29. LORIJN, R. H. W., AND L. D. LONGO. Norepinephrine elevation in the fetal lamb: oxygen consumption and cardiac output. *Am. J. Physiol.* (Regulatory Integrative Comp. Physiol. 8): R115–R122, 1980..

30. MARTIN, A. A., R. KAPOOR, AND G. C. SCROOP. Hormonal factors in the control of heart rate in normoxaemic and hypoxaemic fetal, neonatal and adult sheep. *J. Dev. Physiol.* 9: 465–480, 1987.

31. MCCULLOUGH, R. E., J. T. REEVES, AND R. L. LILJEGREN. Fetal growth retardation and increased infant mortality at high altitude. *Arch. Environ. Health* 32: 36–39, 1977.

32. MCPHERSON, R. A., M. F. KRAMEN, J. M. COVELL, AND W. F. FRIEDMAN. A comparison of the active stiffness of fetal and adult cardiac muscle. *Pediatr. Res.* 10: 660–664, 1976.

33. MEIER, P. R., D. K. MANCHESTER, F. C. BATTAGLIA, AND G. MESCHIA. Fetal heart rate in relation to body mass. *Proceedings of the Society for Experimental Biology and Medicine* 172: 107–110, 1983.

34. MOORE, L. G., S. S. ROUNDS, D. JAHNIGEN, R. F. GROVER, AND J. T. REEVES. Infant birth weight is related to maternal arterial oxygenation at high altitude. *J. Appl. Physiol.* 52: 695–699, 1982.

35. PEETERS, L. L. H., R. E. SHELDON, M. D. JONES, JR., E. L. MAKOWSKI, AND G. MESCHIA. Blood flow to fetal organs as a function of arterial oxygen content. *Am. J. Obstet. Gynecol.* 135:637–66, 1979.

36. RUDOLPH, A. M. Distribution and regulation of blood flow in the fetal and neonatal lamb. *Circ. Res.* 57: 811–821, 1985.

37. RUDOLPH, A. M., AND M. A. HEYMANN. Cardiac output in the fetal lamb: the effect of spontaneous and induced changes of heart rate on right and left ventricular output. *Am. J. Obstet. Gynecol.* 124: 183–192, 1976.

38. RUDOLPH, A. M., AND M. A. HEYMANN. The circulation of the fetus in utero: Methods for studying blood flow. *Circ. Res.* 21: 163–184, 1967.

39. RURAK, D. W. Plasma vasopressin levels during hypoxaemia and the cardiovascular effects of exogenous vasopressin in the foetal and adult sheep. *J. Physiol. Lond.* 277: 341–357, 1978.

40. SHELDON, R. E., L. L. H. PEETERS, M. D. JONES JR., E. L. MAKOWSKI, AND G. MESCHIA. Redistribution of cardiac output and oxygen delivery in the hypoxemic fetal lamb. *Am. J. Obstet. Gynecol.* 135: 1071–1078, 1979.

41. SINGH, S., J. W. SPARKS, G. MESCHIA, F. C. BATTAGLIA, AND E. L. MAKOWSKI. Comparison of fetal and maternal hindlimb metabolic quotients in sheep. *Am. J. Obstet. Gynecol.* 149: 441–449, 1984.

42. TEITEL, D. F., D. SIDI, T. CHIN, C. BRETT, M. A. HEYMANN, AND A. M. RUDOLPH. Developmental changes in myocardial contractile reserve in the lamb. *Pediatr. Res.* 19: 948–955, 1985.

43. THORNBURG, K. L., AND M. J. MORTON. Filling and arterial pressures as determinants of RV stroke volume in the sheep fetus. *Am. J. Physiol.* 244 (*Heart Circ. Physiol.* 13): H656–H663, 1983.

44. THORNBURG, K. L., AND M. J. MORTON. Filling and arterial pressures as determinants of left ventricular stroke volume in fetal lambs. *Am. J. Physiol.* 251 (*Heart Circ. Physiol.* 20): H961–H968, 1986.

45. Walker, A. M., J. R. Cannata, M. H. Dowling, B. C. Ritchie, and J. E. Maloney. Age-dependent pattern of autonomic heart rate control during hypoxia in fetal and newborn lambs. Biol. Neonate 35: 198–208, 1979.

46. WEST, J. B. American medical research expedition to Everest: a study of man during extreme hypoxia. In: *Hypoxia, Exercise and Altitude.* New York, NY: A. R. Liss, p. 431–440, 1983.

CHAPTER TWO

ACTIVATOR CALCIUM AND MYOCARDIAL CONTRACTILITY IN FETAL SHEEP EXPOSED TO LONG-TERM HIGH-ALTITUDE HYPOXIA

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The experimental data in this paper were gathered and analyzed by the first author. Virginia Stiffel, Dr. Gilbert's technician, provided invaluable technical support, especially in collecting the ryanodine binding data, and performing protein assays. The manuscript was written and revised by the first author, then reviewed and edited by Drs. Pearce, Longo, Gilbert, and Hessinger before being sent to the American Journal of Physiology.

Abstract

We studied myocardial contractility in fetal sheep from ewes exposed to ~112 days of hypoxia at high-altitude (3,820 m). We measured the inotropic response to extracellular calcium $[Ca^{2+}]_o$ (0.2-10 mM) and ryanodine (10^{-10}–10^{-4} M) in isometrically contracting papillary muscles, and quantified dihydropyridine (DHPR) and ryanodine (RyR) receptors. In hypoxic fetuses, curves describing the force-$[Ca^{2+}]_o$ relationship were left-shifted, and the top plateaus were decreased by ~35% in both left and right ventricles. In normoxic and hypoxic fetuses, ryanodine (10^{-4} M) reduced maximum active tension (T_{max}) to ~25–40% of baseline values, indicating that the sarcoplasmic reticulum was the chief source of activator calcium, and that calcium influx alone was not sufficient to activate a contraction of normal amplitude. Hypoxia resulted in a lower T_{max} in the right ventricle and lower $\pm dT/dt_{max}$ in the left ventricle following ryanodine. DHPR number did not change, but RyR number in the right ventricle, and the RyR: DHPR ratios in both ventricles were higher in hypoxic fetuses. We conclude that hypoxia decreases contractility, possibly by reducing the availability of activator calcium. Further studies are needed to directly measure the calcium current and intracellular calcium transient, and to examine myofilament protein and ATPase activity.

Index terms: extracellular calcium, inotropy, ryanodine, dihydropyridine receptor, PN200-110

Introduction

The ability of the heart to pump blood depends on four main factors: preload, afterload, heart rate, and contractility or, equivalently, inotropic state. In previous experiments, our lab evaluated the effects of long-term high-altitude hypoxia on ovine fetal right (RV) and left (LV) ventricular performance in vivo (2, 18, 19, 20). In studies of fetal cardiac function during two weeks of hypoxia beginning at 125 days of gestation (2, 19), the RV and LV operated near the plateau of their function curves with little preload reserve in both normoxic and hypoxic fetuses. RV output decreased by 30% after 3 days of hypoxia, but LV output decreased only after 14 days of hypoxia by 21%. In another series of experiments of exposure to hypoxia during days 30–134 of gestation (20), both fetal ventricles operated near the plateau of their function curves with little preload reserve in both normoxic and hypoxic fetuses. The plateau of the right ventricular function curve was significantly lower (~35%) in hypoxic fetuses; however, left ventricular output was not significantly reduced (~85% of controls).

Because the reduction in cardiac performance observed in hypoxic fetuses could not be completely explained by changes in ventricular weights, preload, afterload, or heart rate (2, 3, 4, 18, 19, 20, 23), we hypothesized that myocardial contractility may be decreased during long-term hypoxia. The purpose of this study was to investigate contractility in isolated cardiac muscle taken from normoxic and chronically hypoxic fetuses during the last week of fetal life. Contractility increases with fetal age, particularly during the last week before birth and the first week of neonatal life (5). We reasoned that if long-term hypoxia alters myocardial contractility, the changes would most likely be apparent during the last week of fetal life when inotropic state increases.

On the cellular level, cardiac contractility depends mainly on the amount of activator calcium that reaches the myofilaments, the amount of contractile protein, its calcium affinity, ATPase activity, and the rate of actin-myosin cross-bridge cycling. The inotropic response to changes in extracellular calcium is an important measure of contractility because calcium is central to excitation-contraction coupling and extracellular calcium is the main source of activator calcium in the immature mammalian heart (10, 17, 28, 38). The cardiac sarcoplasmic reticulum and the T-tubule systems are comparatively well developed in the late-term sheep fetus (33, 34), suggesting that the SR may provide a significant fraction of the activator calcium. In this study, we characterized the relationship between extracellular calcium and cardiac contractile force, and determined the extent to which steady-state contractile activity was dependent on ryanodine sensitive calcium (SR) stores. In addition, we determined whether changes in the inotropic response to calcium was related to number of dihydropyridine and ryanodine receptors.

Methods

We obtained 32 time-dated pregnant ewes of a mixed western breed from a single supplier (Nebeker Ranch, Lancaster, CA) and randomly separated them into control and long-term hypoxic groups. The control group (n=16) remained at Nebeker Ranch (altitude ~760 m) until 138–142 days gestation. At 30 days gestation, we transported the long-term hypoxic group (n=16) to the Barcroft Laboratory, White Mountain Research Station (WMRS, Bishop, CA; altitude 3,820 m, barometric pressure ~480 Torr) where they remained until 138–142 days gestation. At both locations, the ewes were kept in a

sheltered pen, and were provided alfalfa pellets, mineral supplements, and clean water *ad libitum*. We transported (~7h trip) animals from either group to our laboratory at Loma Linda University where either they underwent immediate study or, in the case of hypoxic ewes awaiting study, a nonocclusive tracheal catheter was surgically implanted (14) so that N_2 gas could be administered to reestablish hypoxemia immediately after arrival at our laboratory. Experiments were scheduled so that fetuses were 142 d gestation on the experimental day. On the experimental day, the ewes were sedated intravenously with thiamylal (10 mg/kg), intubated, and kept under deep surgical anesthesia (Halothane 5% in O_2), while we delivered the fetuses through a midline laparotomy. Within 30 seconds, we removed the fetal hearts via midline thoracotomy and placed them in warm (39 °C), heparinized, low-calcium (0.2 mM Ca^{2+}) Tyrode solution continuously bubbled with 95% O_2–5% CO_2.

Tissue Preparation

Contractile force in isolated muscle. For the contractile studies, we excised four thin papillary muscle strips (0.3–1.1 mm diameter) or trabeculae carnae (0.3–0.6 mm diameter) from the fetal left (LV) and right (RV) ventricles then tied a loop of fine suture to each end. One end of the muscle was attached to a hook, whose position could be varied to adjust tension, and the other to a stainless steel wire attached to an isometric force transducer (Grass Inst., model FT03, Quincy, MA). The muscles were placed in a water-jacketed 10 ml bath (Radnoti Glass, Monrovia, CA), stretched to their just-taut length, stimulated electrically (as described below), and allowed to equilibrate for ~1 h in warm (39±0.1 °C) Tyrode solution continuously bubbled with 95% O_2-5% CO_2. Under these conditions, typical values for PO_2 and PCO_2 in the bath were ~550 and ~32 Torr,

respectively. The Tyrode solution contained (in mM): 2.0 $CaCl_2$, 140 NaCl, 20 $NaHCO_3$, 6 KCl, 1 $MgCl_2$, 10 glucose, and 5 HEPES (N-2-Hydroxyethyl piperazine-N′-2-ethanesulfonic acid), pH 7.40±0.02. When the muscle response stabilized (30–40 min.), we gradually stretched the muscles to the length at which active tension was maximal, L_{max}, and maintained that length for the duration of the experiment. Typically, between 2–3 grams of preload were required to achieve L_{max}. At the end of the experiment, while the muscle was still stretched to L_{max}, we measured muscle diameter optically using an eyepiece reticle with graduations of 0.1 mm. The average of four measurements taken at equal intervals along the length of the muscle was used as the diameter. To calculate cross-sectional area, we assumed cylindrical geometry.

A pair of platinum electrodes for field stimulation was incorporated into the muscle holder. We stimulated the muscles with a 7 ms square-wave pulse at 0.8 Hz (Grass Inst., model S/88). In preliminary experiments, we determined that 0.8–1 Hz was an optimal rate of stimulation, which allowed diastolic tension to return to resting levels well before the onset of the next contraction and produced stable contractions with a minimum of rundown. Any muscle that had an unusually high threshold voltage (>10 V in our system) at baseline, demonstrated a spontaneous increase in resting tension (i.e. contracture) at any time during the experiment, showed rapid run-down (>5% after 1h), or contracted intermittently was excluded from the study.

We recorded each contraction on an eight-channel polygraph (Gould Electronics model RS3800, Cleveland, OH). Simultaneously, we used the microcomputer program for real time data acquisition (RTD) developed in our laboratory to sample the analog signals at a rate of 512 Hz. The calibrated digital values were evaluated by the contraction

pattern recognition algorithms in RTD to determine maximum active tension (T_{max}; g), time from onset to peak tension (sec), duration of contraction (sec), and maximum rates of rise ($+dT/dt_{max}$; $g \cdot sec^{-1}$) and fall ($-dT/dt_{max}$; $g \cdot sec^{-1}$) in tension. Relaxation time was calculated on-line as the difference between duration of contraction and time to peak tension. The resulting values were stored on magnetic hard disk for later analysis.

Dihydropyridine and ryanodine receptor assays. For the radioligand binding assays, we cut sections (~2 cm square) from the right and left ventricular free walls, rinsed them in heparinized Tyrode solution, blotted them with cotton gauze pads to remove excess fluid, then froze them in liquid nitrogen. We stored the tissue in sealed vials at -70 °C until assay.

Experimental Protocol

Contractile force and extracellular calcium. After the muscles were equilibrated (~1 h), we replaced the perfusate with Tyrode solution containing 0.2 mM Ca^{2+}, then added calcium chloride (400 mM stock in 5 mM HEPES) in a series of small aliquots to construct a cumulative dose-response curve between 0.2 mM and 10.0 mM. We allowed the muscles to stabilize after each addition of $CaCl_2$ (5-10 minutes), and at the plateau of each new steady-state recorded 20 contractions for later analysis. As we increased $[Ca^{2+}]_o$ above 6–8 mM, the muscles became refractory to stimulation. Although we increased stimulus voltage, we could not increase calcium concentration beyond 10 mM without developing contracture in most muscles. Thus, only the data collected between 0.2 mM and 10 mM were used in the data analysis as outlined below.

Contractile force and the sarcoplasmic reticulum as a source of activator calcium. We selected a separate set of muscles, and after the ~1 h equilibration, recorded baseline

control values to which all subsequent measurements were compared. Thus, each muscle served as its own control. We then added small aliquots of ryanodine (Calbiochem), which interferes selectively and irreversibly with the release of calcium from the sarcoplasmic reticulum (7, 27, 32), to construct a cumulative dose-response curve between 10^{-10} to 10^{-4} M. We allowed the muscles to equilibrate after each addition of ryanodine (~20 minutes), and at the plateau of each new steady-state recorded 20 contractions for later analysis.

Ligand Binding Assays. On the assay day, we homogenized 1–2 grams of tissue in 0.25 M sucrose, 20 mM Tris, pH 7.4 with a Polytron (Brinkman Instruments, Westbury, NY). The homogenizing buffer also contained a cocktail of protease inhibitors: 76.8 nM aprotinin, 0.83 mM benzamidine, 1 mM iodoacetamide, 1.1 mM leupeptin, 0.7 mM pepstatin-A, and 0.23 mM PMSF (phenylmethylsulfonyl fluoride). The homogenate was spun for 45 minutes at 110,000 g (50,000 rpm) in an ultracentrifuge (Beckman Instruments Model L3-50) equipped with a Ti-50 rotor; temperature was maintained at 4 °C. We decanted the supernatant, and resuspended the pellet in buffer containing 50 mM Tris, pH 7.4 and the protease inhibitor cocktail for use in the binding assays. [^{3}H] (+)-PN200-110 and [^{3}H] Ryanodine (New England Nuclear) were used to estimate the number of dihydropyridine receptors (DHPR) and SR Ca^{2+}-release channels (RyR) present in the fetal heart, respectively. Resuspended pellet protein was adjusted to a concentration of 0.3 mg/ml in a final volume of 300 µl. For DHPR, specific binding was carried out in the presence of 0.01–10 nM [^{3}H] (+)-PN200-110, 50 µM nifedipine, 25 mM Tris, 150 mM KCl, 10 mM HEPES, 2 mM $MgCl_2$, 0.2 mM $CaCl_2$ at pH 7.4. Triplicate samples were incubated at room

temperature for 90 minutes in the dark. Membranes were rinsed three times with 3 ml ice-cold 10 mM Na-HEPES, pH 7.4, and collected by vacuum filtration on Whatman GF/C filters (Whatman Biosystems Ltd., UK). For RyR, specific binding was carried out in the presence of 1–200 nM [^{3}H] Ryanodine, 33 μM cold ryanodine, 25 mM Tris, 1 M NaCl, 5 mM AMP, 20 mM HEPES, 0.5 mM $CaCl_2$, pH 7.4. Duplicate samples were incubated at 37 °C for 90 minutes. Membranes were rinsed three times with 3 ml distilled water and collected by vacuum filtration on Whatman GF/C filters. The filters from both binding assays were placed in scintillation vials containing 6 ml Beckman Ready Protein$^+$ scintillation cocktail, and radioactivity was measured in a Beckman Liquid Scintillation Counter. Protein was determined by the Lowry method (26).

In preliminary experiments (data not shown) we determined that the conditions outlined above were optimal for binding. In particular, [^{3}H] (+)-PN200-110 binding required high potassium concentrations (150 mM KCl), suggesting that membrane depolarization was important for binding. Binding in the presence of 145 mM NaCl yielded inconsistent results. [^{3}H] Ryanodine binding was sensitively dependent on calcium concentration, with maximum binding occurred between 0.3-1 mM. Optimal binding required 1 M NaCl, 5 mM AMP, and 0.5 mM $CaCl_2$.

Data Analysis

Contractile force and extracellular calcium. For each fetus, we averaged the 20 contractions recorded at each concentration of calcium, corrected the values for muscle cross-sectional area, and plotted the results against log $[Ca^{2+}]_o$. We fit the data to Hill curves using the non-linear regression analysis algorithms in GraphPAD Prism (GraphPAD Software, San Diego, CA). The resulting top plateau of each sigmoid curve

was used as the calculated maximum response for that fetus. Pooled data from normoxic and hypoxic groups were used to fit the curves displayed in Fig. 1, and to determine the EC_{50} values in Table 1. To describe sensitivity, the response at each concentration of calcium was expressed as a percentage of the individual calculated maxima. Pooled data from normoxic and hypoxic groups were then used to fit the curves displayed in Fig. 2, and to determine the EC_{50} values in Table 1.

Contractile force and functional sarcoplasmic reticulum. Baseline values were time-averaged and designated the 100% value (Table 2). For each fetus, the 20 contractions recorded at each concentration of ryanodine were averaged, expressed as a percentage of the baseline value, and plotted against log [Ryanodine]. The resulting data were fit to Hill curves using the non-linear regression analysis algorithms in Prism. Pooled data from hypoxic and normoxic groups were then used to fit the curves displayed in Fig. 3, and for the IC_{50} values listed in Table 3.

Dihydropyridine and ryanodine receptor assays. The radioactivity measured in duplicate and triplicate samples was averaged to produce a single value for each concentration of the radioligand. Raw counts were converted to femtomoles of receptor per milligram of protein, and the resulting data were fit to a rectangular hyperbola using the non-linear regression analysis algorithms in Prism. Pooled data from normoxic and hypoxic fetuses were used to fit the curves displayed in Figures 4 and 5. The resulting B_{max} and K_D values are reported in Table 4. The ryanodine receptor: dihydropyridine receptor ratios (Table 5) were determined by dividing the B_{max} for [^{3}H] Ryanodine by the B_{max} for [^{3}H] (+)-PN200-110.

Statistics

The dose-response curves for calcium and ryanodine, were analyzed in SPSS (SPSS Inc., Chicago, IL) using doubly multivariate repeated measures analysis of variance for a split-plot design. In their respective analyses, calcium and ryanodine concentrations were within-subjects factors with 12 and 8 levels, respectively. Ventricle was a within-subjects factor with 2 levels (LV and RV), and oxygen was the between-subjects factor with 2 levels (hypoxia or normoxia). $T_{max}$and $\pm dT/dt$ were the measures. EC_{50} (or IC_{50}) values were compared using Student's *t*-test. For the radioligand binding assays, B_{max} and K_D values were compared using Student's *t*-test. For all comparisons, statistical significance was set at $P<0.05$. Results were expressed as means ± SE.

Results

Extracellular calcium concentration and contractility

Under control conditions (2.0 mM Ca^{2+}), T_{max}, $\pm dT/dt_{max}$, and the time course of contraction were similar in normoxic and hypoxic fetuses. However, relaxation time was prolonged significantly in the hypoxic left ventricle (332±11 msec vs. 280±13 msec). As extracellular calcium concentration was varied between 0.2 mM–10 mM, contractile force increased in a dose-dependent manner (Fig. 1). Resting (diastolic) tension remained low, indicating that calcium uptake and extrusion mechanisms functioned adequately. Chronic hypoxia significantly decreased the maximum inotropic response to calcium. The magnitude of the effects were similar in both ventricles (~35% reduction) with the exception of $\pm dT/dt_{max}$ in the LV which was reduced by ~70% (Fig. 2C). When the dose-response data were expressed as a percentage of the maximum response, the

Figure 1. Relationship between extracellular calcium concentration ($[Ca^{2+}]_o$) and maximum active tension (T_{max}), maximum rate of rise in tension ($+dT/dt_{max}$), and maximum rate of relaxation ($-dT/dt_{max}$) in the left (Panels A–C) and right (Panels D–F) ventricles from normoxic (■) and chronically hypoxic (□) fetuses.

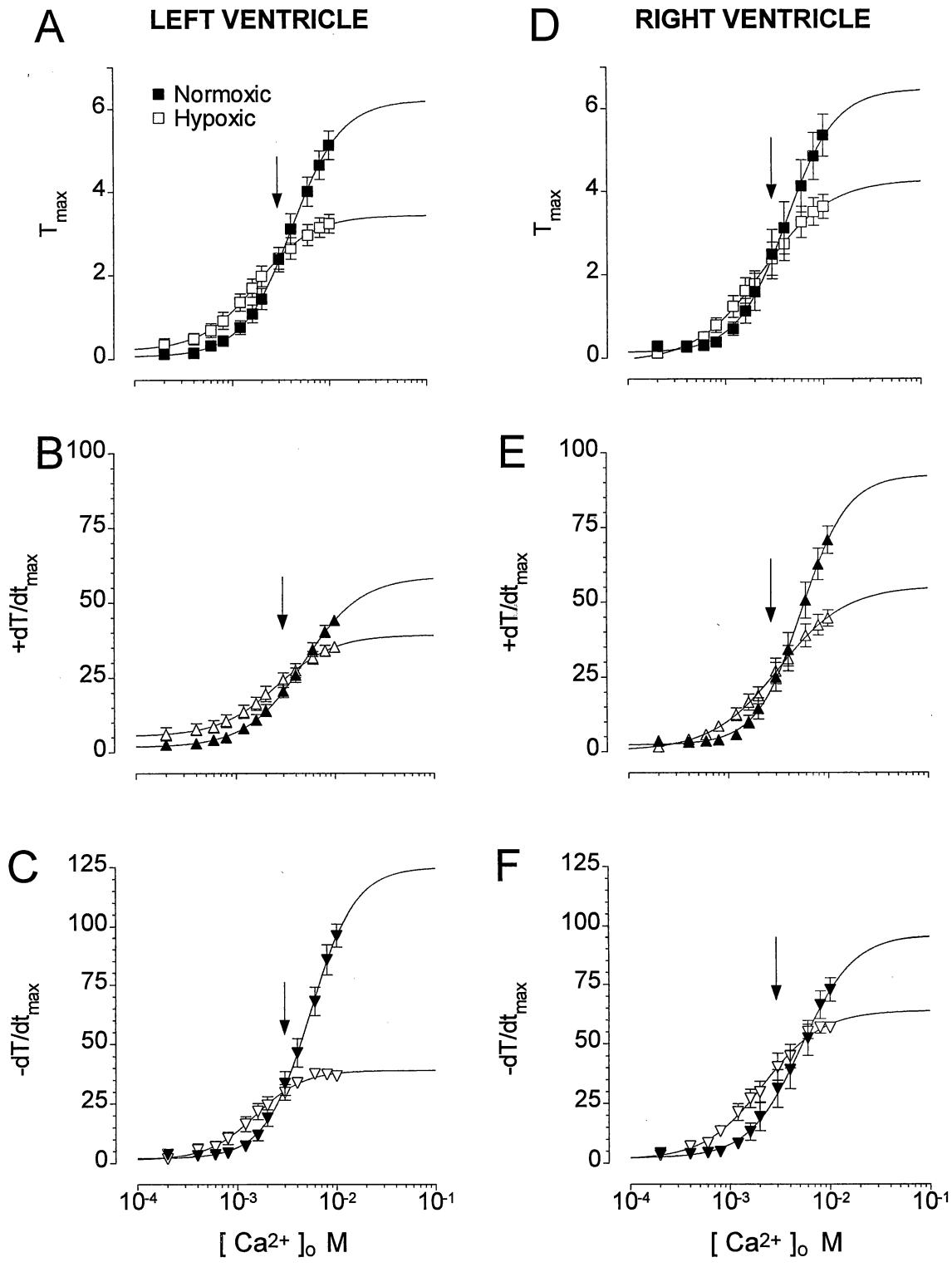
A
LEFT VENTRICLE
D
RIGHT VENTRICLE
Normoxic
Hypoxic
T max
+dT/dt max
-dT/dt max
B
E
C
F
[Ca2+]o M

Figure 2. The force-extracellular calcium sensitivity curves for papillary muscle from normoxic (■) and hypoxic (□) fetuses, expressed as a percentage of the maximum response shown in figure 1. Maximum active tension (T_{max}), maximum rate of rise in tension ($+dT/dt_{max}$), and maximum rate of relaxation ($-dT/dt_{max}$). The arrows indicate the points at 3.1 mM calcium, the normal fetal serum calcium concentration

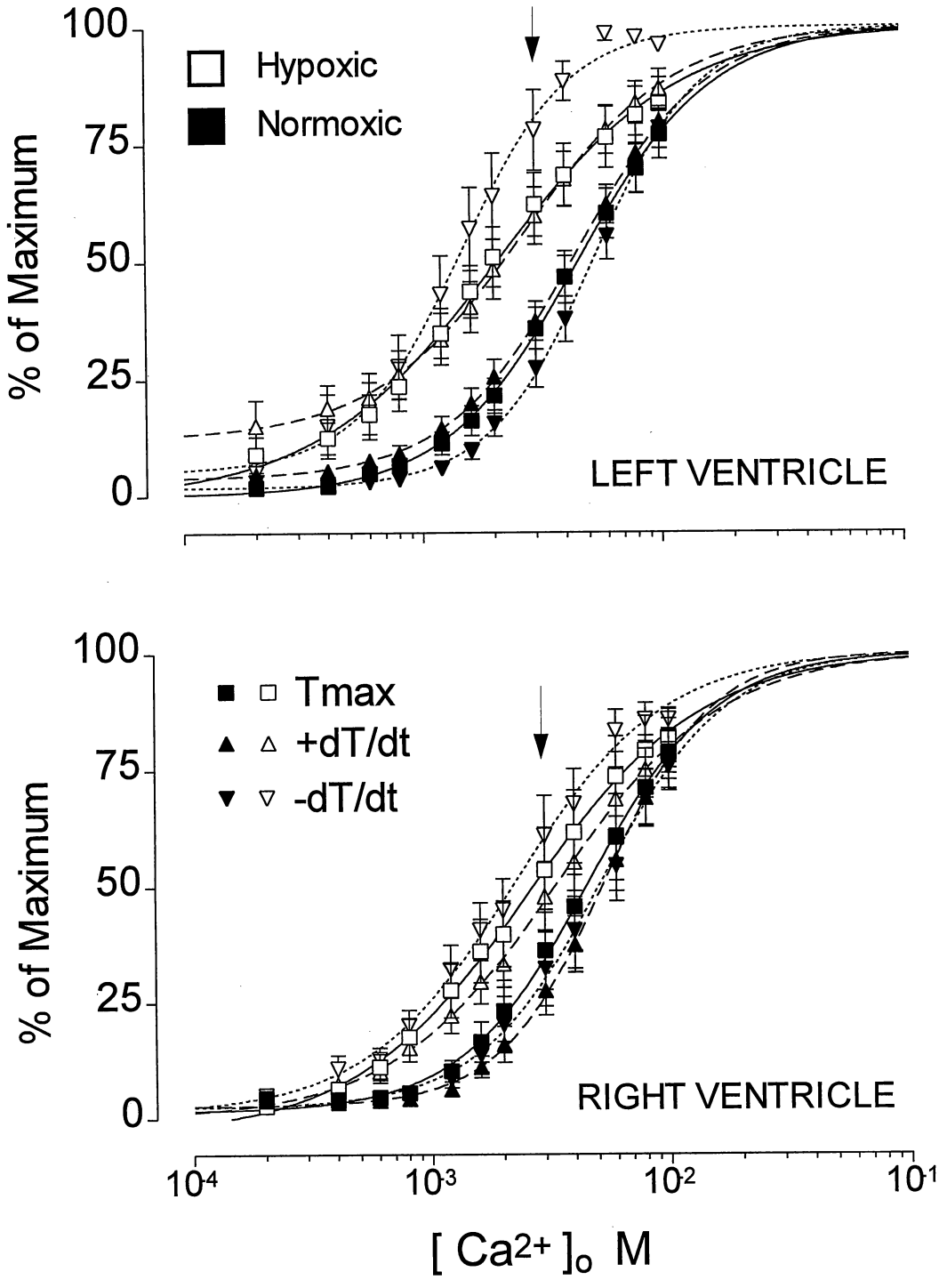
100
75
50
25
0
% of Maximum
Hypoxic
Normoxic
LEFT VENTRICLE
Tmax
+dT/dt
-dT/dt
RIGHT VENTRICLE
10-4
10-3
10-2
10-1
[Ca2+]o M

TABLE 1. EC_{50} values for the force-extracellular calcium relationship in isolated papillary muscles.

	LEFT VENTRICLE		RIGHT VENTRICLE	
Variable	**Normoxic**	**Hypoxic**	**Normoxic**	**Hypoxic**
T_{max}	4.53 ± 0.06	2.06 ± 0.13*	4.56 ± 0.1	2.54 ± 0.09*
$+dT/dt_{max}$	4.46 ± 0.04	2.70 ± 0.05*	5.37 ± 0.06	3.32 ± 0.07*
$-dT/dt_{max}$	5.31 ± 0.06	1.48 ± 0.06*	5.20 ± 0.13	2.23 ± 0.12*

Extracellular calcium concentration (in mM) at which contractile activity was half-maximal (EC_{50}) from the curve fits in Figures 1 and 2. Values are means ± SE; N=8 in each group. *P <0.01 compared with normoxic.

calcium sensitivity curves in hypoxic fetuses were significantly left-shifted when compared to the corresponding curves for normoxic fetuses (Fig. 2). As a result, the EC_{50} values (Table 1) were ~1.8–3.8 mM lower in hypoxic ventricles than in the corresponding normoxic ventricles.

Calcium release from the SR

After the 1h equilibration period in normal Tyrode solution (2.0 mM Ca^{2+}), baseline contractile values were not significantly different between normoxic and hypoxic fetuses (Table 2). As the ryanodine concentration was varied between 10^{-10} M–10^{-4} M, contractile force decreased in a dose-dependent manner (Fig. 3). In normoxic fetuses, at 10^{-4} ryanodine Tmax was reduced to ~45% of baseline values in both left and right ventricles. $+dT/dt_{max}$ was reduced to 30% and 8%, while $-dT/dt_{max}$ was reduced to 30% and 12% in the left and right ventricle, respectively. Resting (diastolic) force remained low and stable after treatment with ryanodine, indicating that cytoplasmic calcium concentrations were not rising.

Qualitatively, the response to ryanodine in hypoxic fetuses was similar to that in normoxic fetuses, indicating that the SR was the chief source of activator calcium in both groups (Fig. 3). However, in the right ventricle, hypoxia potentiated the ryanodine effect on T_{max} but not on $\pm dT/dt_{max}$ (Fig. 3D-F). In the left ventricle, hypoxia did not change the T_{max} response, but potentiated the negative-inotropic effect of ryanodine on $\pm dT/dt_{max}$ (Fig. 3B, C). Hypoxia did slightly, but significantly, decrease the sensitivity of both ventricles to ryanodine, as evidenced by the small increase in IC_{50} values (Table 3).

TABLE 2. Baseline contractile values for isolated papillary muscles before adding ryanodine.

	LEFT VENTRICLE		RIGHT VENTRICLE	
	Normoxic	**Hypoxic**	**Normoxic**	**Hypoxic**
T_{max}	1.43 ± 0.24	1.98 ± 0.40	1.58 ± 0.41	1.77 ± 0.20
$+dT/dt_{max}$	14.08 ± 1.86	19.63 ± 3.92	14.46 ± 3.11	18.83 ± 5.24
$-dT/dt_{max}$	19.04 ± 3.07	24.37 ± 4.90	19.38 ± 4.50	29.76 ± 8.90

Raw values of peak active tension (T_{max}, $g \cdot mm^{-2}$), peak rate of rise in tension ($+dT/dt_{max}$, $g \cdot sec^{-1} \cdot mm^{-2}$) and peak rate of relaxation ($-dT/dt_{max}$, $g \cdot sec^{-1} \cdot mm^{-2}$) at 2.0 mM Ca^{2+}. Values are means ± SE; N=9 in each group. These values were the baseline (100%) for the curves in Figure 3. Although hypoxic values tended to be ~30% higher, the differences were not statistically significant.

Figure 3. The negative inotropic effect of ryanodine on T_{max}, $+dT/dt_{max}$, and $-dT/dt_{max}$ in papillary muscles from the left (Panels A–C) and right (D–F) ventricles of normoxic (■) and hypoxic (□) fetuses.

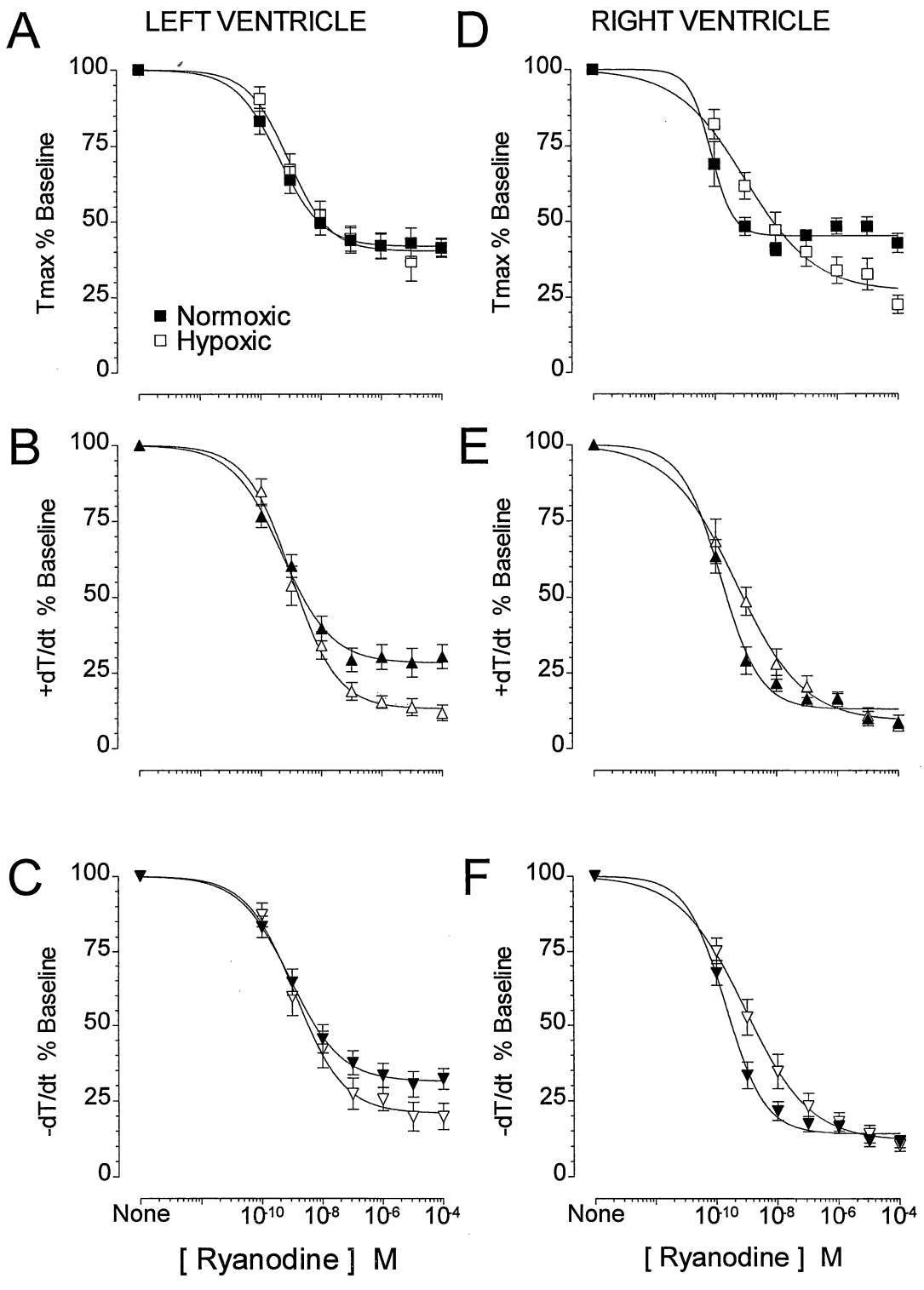
A
LEFT VENTRICLE
D
RIGHT VENTRICLE
Tmax % Baseline
100
75
50
25
0
Normoxic
Hypoxic
B
E
+dT/dt % Baseline
C
F
-dT/dt % Baseline
None
10-10
10-8
10-6
10-4
[Ryanodine] M

TABLE 3. IC_{50} values for the ryanodine dose-response curves.

	LEFT VENTRICLE		RIGHT VENTRICLE	
Variable	**Normoxic**	**Hypoxic**	**Normoxic**	**Hypoxic**
T_{max}	0.44 ± 0.02	0.93 ± 0.02*	0.08 ± 0.02†	1.11 ± 0.38*
$+dT/dt_{max}$	0.50 ± 0.09	1.04 ± 0.15*	0.15 ± 0.03†	0.48 ± 0.08*†
$-dT/dt_{max}$	0.86 ± 0.07	1.26 ± 0.23	0.19 ± 0.03†	0.82 ± 0.11*

Ryanodine concentration (in nM) at which the inhibitory effect was half-maximal (IC_{50}) from the curve fits in Figure 3. Values are means ± SE; N=9 in each group. *P <0.05 compared with normoxic. †P <0.05 compared with left ventricle.

Quantification of dihydropyridine and ryanodine receptors

Figures 4 and 5 show specific binding of [^{3}H] (+)-PN200-110 and [^{3}H] ryanodine, respectively, as a function of ligand concentration. For both ligands, the data fit a single class of high-affinity binding sites. The resulting B_{max} and K_D values are shown in Table 4. For [^{3}H] (+)-PN200-110 binding (Fig. 4), B_{max} was not different in either ventricle of normoxic and hypoxic fetuses (Table 4), indicating that hypoxia did not reduce the number of dihydropyridine receptors. In hypoxic fetuses, B_{max} for [^{3}H] ryanodine was significantly higher in both the left and right ventricles (Fig. 5 and Table 4). In addition, the RyR:DHPR ratios were ~23% and 25% higher in the hypoxic LV and RV, respectively, than in normoxic controls (Table 4).

Discussion

Extracellular calcium and contractility

To determine maximum contractile force, we elevated extracellular calcium concentration. Elevating $[Ca^{2+}]_o$ increases the influx of calcium (30), which, in turn, increases the amount of calcium stored in and released from the sarcoplasmic reticulum (13, 39) and increases the calcium delivery of calcium to the myofilaments.

In normoxic fetuses, maximum contractility was ~4 to 8-fold higher than baseline levels (Fig. 1). This represents a significant inotropic reserve. Long-term hypoxia decreased inotropic reserve in two ways. In hypoxic fetuses, maximum contractility decreased by ~35% in both left and right ventricles (Fig. 1). At the same time, the force-$[Ca^{2+}]_o$ curves were left-shifted (Figs. 1&2), so that their EC_{50} values were reduced

Figure 4. Specific binding of [^{3}H] (+)-PN200-110 to homogenates from the left (A) and right (B) ventricles of normoxic (■) and hypoxic (□) fetuses.

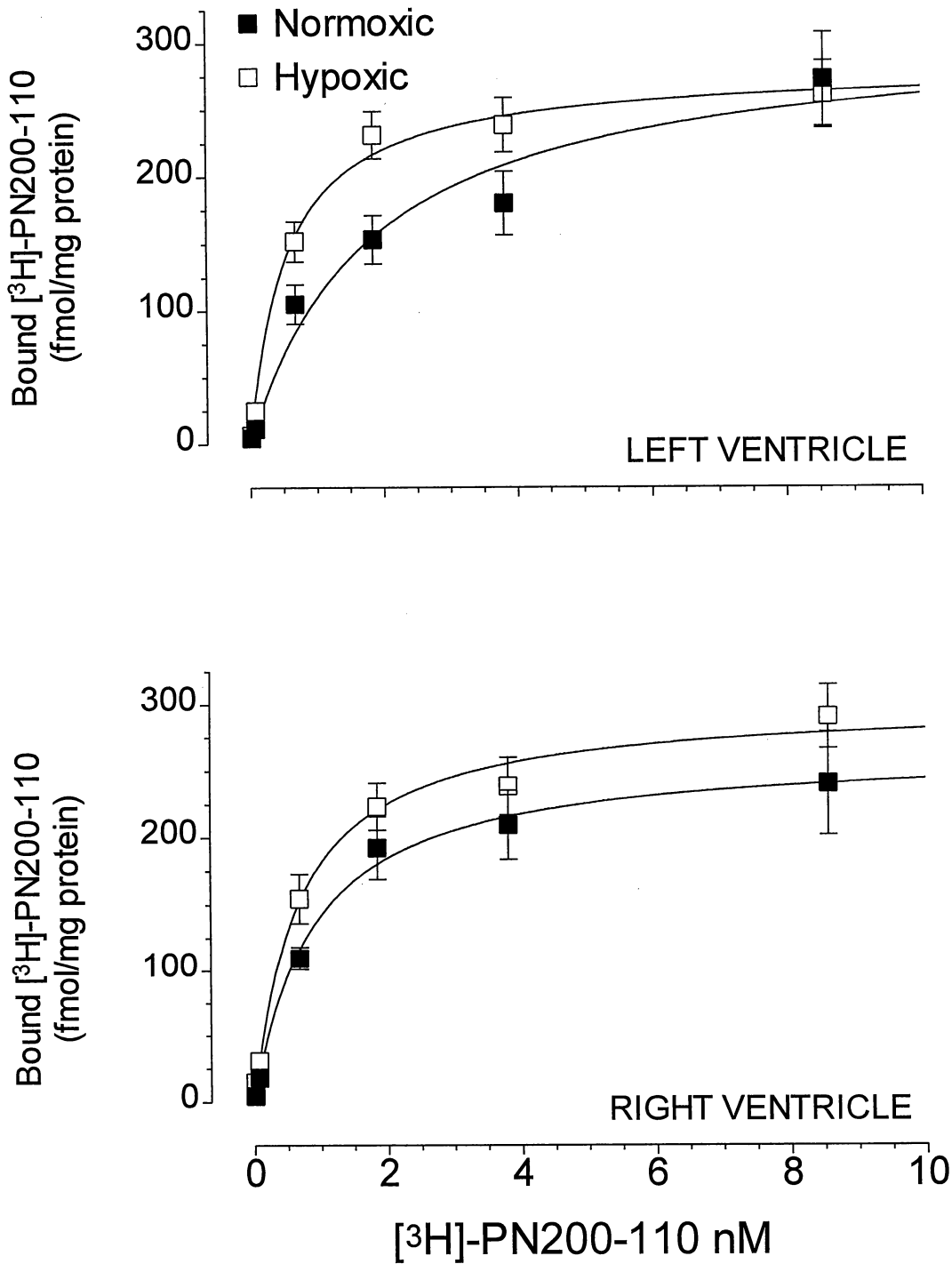
Normoxic
Hypoxic
Bound [3H]-PN200-110 (fmol/mg protein)
300
200
100
0
LEFT VENTRICLE
Bound [3H]-PN200-110 (fmol/mg protein)
300
200
100
0
RIGHT VENTRICLE
0
2
4
6
8
10
[3H]-PN200-110 nM

Figure 5. Specific binding of [^{3}H] Ryanodine to homogenates from the left (A) and right (B) ventricles of normoxic (■) and hypoxic (□) fetuses.

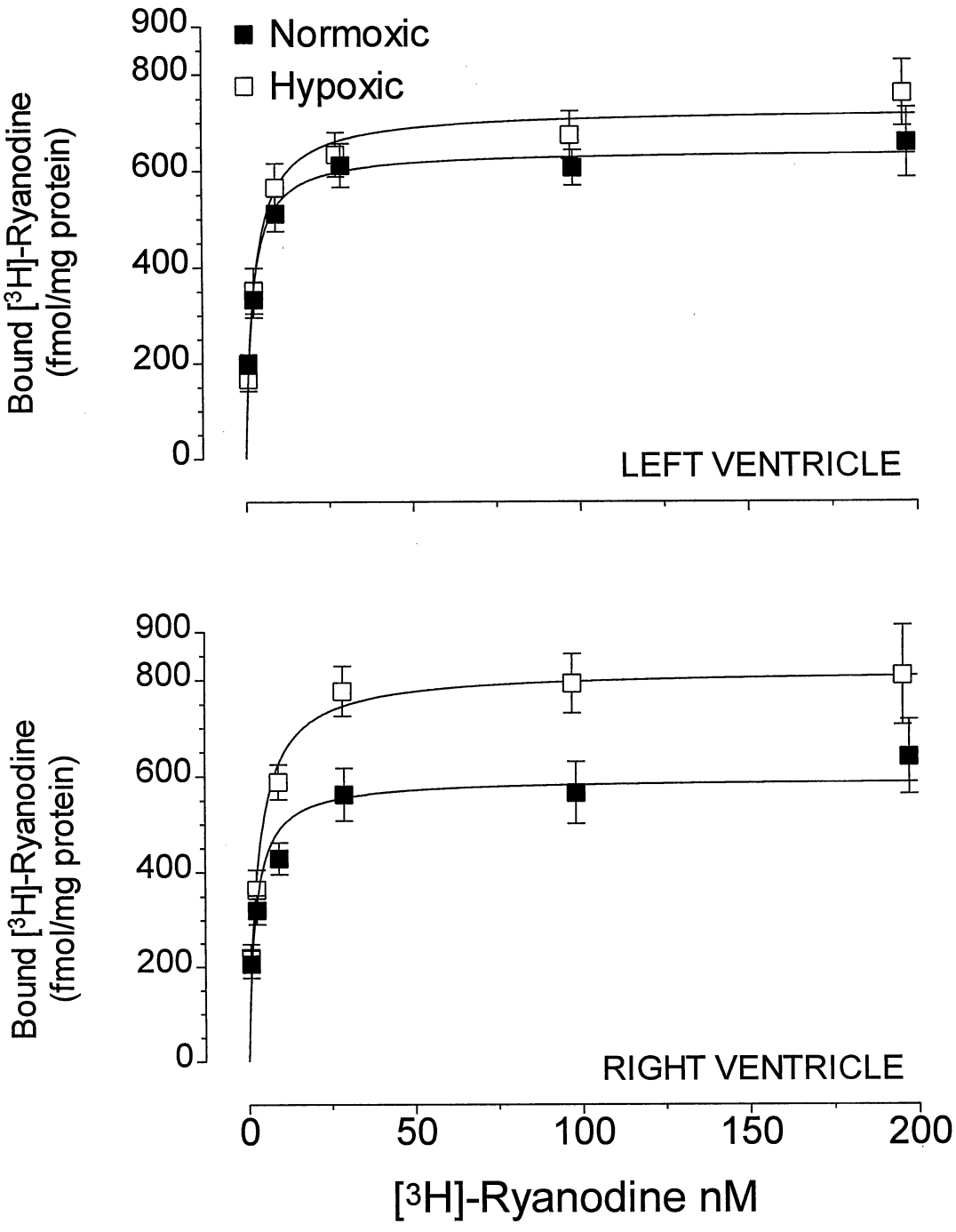
Normoxic
Hypoxic
Bound [3H]-Ryanodine (fmol/mg protein)
900
800
600
400
200
0
LEFT VENTRICLE
Bound [3H]-Ryanodine (fmol/mg protein)
900
800
600
400
200
0
RIGHT VENTRICLE
0
50
100
150
200
[3H]-Ryanodine nM

TABLE 4. Dihydropyridine and ryanodine receptors in fetal sheep at 142 days gestation.

	LEFT VENTRICLE			RIGHT VENTRICLE		
	B_{MAX}	K_D	RyR:DHPR	B_{MAX}	K_D	RyR:DHPR
[^{3}H] (+)-PN200-110						
Normoxic	308 ± 37	1.82 ± 0.63	——	265 ± 9	0.87 ± 0.12	——
Hypoxic	280 ± 9	0.54 ± 0.08*	——	293 ± 19	0.62 ± 0.18	——
[^{3}H] Ryanodine						
Normoxic	640 ± 16	2.03 ± 0.29	2.08	593 ± 29	2.01 ± 0.56	2.24
Hypoxic	715 ± 42*	2.33 ± 0.77	2.55	819 ± 24*†	2.84 ± 0.45	2.80

Dihydropyridine (DHPR) and ryanodine (RyR) receptors, B_{MAX} (fmol·mg^{-1} protein), in membranes from normoxic and hypoxic fetal hearts; concentration of radioligand at which binding was half-maximal, K_D (nM). Values are means ± SE of group data; N=10 in each group. *P <0.01 compared with normoxic. †P <0.001 compared with left ventricle.

(Table 1). As a result, when $[Ca^{2+}]_o$ was increased above 2.0 mM, contractility saturated rapidly, increasing by only ~1.5 to 2-fold over baseline values. Because the force-$[Ca^{2+}]_o$ curves were left-shifted and the top plateaus were lower, hypoxic fetuses had limited inotropic reserve (Fig. 1). However, values for baseline contractile parameters in hypoxic fetuses at 2.0 mM Ca^{2+} were not significantly different from those in normoxic fetuses (Table 2). Thus, when baseline T_{max} was expressed as a percent of the maximum response, baseline contractility was ~20% of maximum in normoxic left and right ventricles, but was ~55% and ~40% of maximum in the hypoxic left and right ventricles, respectively, indicating reduced contractile reserve in the hypoxic hearts. Teitel, et al (36)found a limited contractility reserve in 1 week old lambs compared to 1 month old lambs, which they attributed to a high β-adrenergic state in the younger animals. Because our studies were done in isolated muscles, β-adrenergic state was not a factor in the lower contractility reserve in the hypoxic fetuses. Rather, the difference in reserve would most likely be explained changes in Ca^{2+} delivery to the myofilaments, Ca^{2+} sensitivity of the myofilaments, or cellular content of myofilaments. At present we do not know which of these possibilities is responsible for the changes observed.

The changes in myocardial contractility and calcium sensitivity in our study were qualitatively similar to the changes reported during cardiac development in several mammalian species. Several investigators (8, 17, 28) have shown that maximum force of contraction is lower in the fetus than in the neonate, and both fetal and neonatal values are less than those in the adult. In addition, the half-maximal $[Ca^{2+}]_o$ (EC_{50}) for $+dT/dt_{max}$ (8, 17, 28), and the threshold for calcium-induced calcium release (12) were both lower in adults than in fetuses or neonates of the same species. The investigators

concluded that contractile force and the sensitivity to extracellular calcium are related to calcium influx, sarcoplasmic reticulum function, and to myofibrillar content and ATPase activity. Those conclusions are consistent with our hypotheses. Furthermore, it has been shown that short-term hypoxia has direct effects on the contractile apparatus of the intact heart (15), on action potential duration (9, 35), and on SR Ca^{2+} uptake and content (1, 35, 40). Further studies are needed to determine whether similar changes occur with exposure to long-term high-altitude hypoxia.

Calcium release from the SR

We treated a group of muscles with ryanodine (7, 24, 27, 32) to determine whether the extracellular calcium influx was sufficient to activate a contraction of normal amplitude, and to estimate the fraction of steady-state contractile activity that depends on calcium released by the sarcoplasmic reticulum. In adult sheep, ryanodine has been shown to "lock" the SR calcium release channel in a fixed open state with low conductance (16, 25), thereby depleting the pool of releasable calcium. Several investigators have shown that active tension, and $\pm dT/dt_{max}$ decline after treatment with ryanodine (7, 24, 27, 29). The extent of decline is thought to reflect the fraction of activator calcium contributed by the SR. Residual tension is thought to reflect the fraction of activator calcium that can be supplied by the influx of extracellular calcium in the absence of a functional SR.

Longitudinal studies of cardiac ultrastructural development from early fetal life to adulthood suggest that cardiac myocytes in the sheep fetus are morphologically and morphometrically more mature than myocytes from the rat, dog, cat, and rabbit (33, 34). Smolich, 1987 (34) showed that cardiac myocytes in the sheep fetus have both sarcolemmal invaginations, which are involved in the formation of T-tubules, and a

meshwork of sarcoplasmic reticulum around the myofilaments by ~115 days gestation. Given the development of M-bands (an indicator of myofibrillar maturation) before birth (33), the presence of identifiable T-tubules and SR on electron micrographs (33, 34), and a high myofibrillar volume density (33), we hypothesized that the late-term fetal sheep heart may depend on its SR to contribute a significant fraction of activator calcium, and, therefore, would be quite sensitive to ryanodine.

Our results indicate that the fetal sheep heart is very sensitive to ryanodine (Fig. 3 and Table 3), and that extracellular calcium influx alone was not sufficient to activate a contraction of normal amplitude. Despite low IC_{50} values (0.08–1.26 nM), fairly high concentrations of ryanodine (~0.5–100 μM) were needed to completely deplete the pool of releasable calcium.

In normoxic fetuses, calcium influx during ryanodine provided enough activator calcium to support a contraction whose T_{max} was ~40–45% of baseline values in both left and right ventricle (Fig. 3). Values for $\pm dT/dt_{max}$ were decreased to between ~8% and ~30% of baseline values, suggesting that the rate at which calcium activated the myofilaments, and the rate at which calcium was removed were markedly reduced during ryanodine. Although we cannot state precisely how much activator calcium is released by the SR in normoxic fetuses, it seems that a reasonable estimate is that at least ~50–92% of beat to beat calcium is supplied by the SR.

In hypoxic fetuses, residual T_{max} after treatment with ryanodine was similar to that in the normoxic left ventricle, ~40% of baseline values (Fig. 3). However, in the hypoxic right ventricle T_{max} was reduced to ~25% of baseline values, a level significantly lower than that in the normoxic right ventricle. The lower residual Tmax in the hypoxic right

ventricle after treatment with ryanodine suggests that calcium release from and/or the amount of calcium stored in the SR may also be reduced under hypoxic baseline conditions.

Baseline contractility (Table 2) tended to be higher in hypoxic fetuses (a consequence of the left-shifted force-$[Ca^{2+}]_o$ curves as discussed above). If myofilament sensitivity to calcium were increased in hypoxic fetuses (resulting in the left-shifted force-$[Ca^{2+}]_o$ curves), then the increased sensitivity may offset a smaller calcium influx and a smaller calcium transient, so that the contraction amplitude (T_{max}) may be normal or increased. This interpretation is consistent with the observation that the force-[Ryanodine] curves were slightly right-shifted in hypoxic fetuses (Fig. 3), giving rise to a small, but statistically significant, increase in IC_{50} values (Table 3). This suggests that the differences in the force-$[Ca^{2+}]_o$ relationship may be related to differences in both calcium handling and myofilament sensitivity to calcium. Further studies are needed to more deeply investigate these possibilities.

Some reports indicate that calcium-induced calcium release (CICR) is decreased following global ischemia or anoxia (35). Others (31) have reported a decrease in Ca^{2+} release from SR release channels following a reduction in pH, which may occur with hypoxia. However, other investigators have reported that CICR is not decreased during short-term hypoxia, but that the rate of calcium uptake into the SR and the amount of calcium stored in the SR are markedly reduced (1, 15, 40). The inhibition of the SR calcium pump is thought to be a result of increased inorganic phosphate and ADP concentrations, which also decrease myofilament sensitivity to calcium (21). This would argue against our interpretation. However, after exposure to long-term hypoxia, no

decrease in cardiac output was observed until 3 days after the onset of hypoxia (2, 19). In a study of cardiac myocyte adaptations to chronic hypoxia, Webster and Bishopric (37) reported a decrease in contractility over a period of 3 days that paralleled a progressive fall in cAMP. This suggests that the mechanisms responsible for decreased cardiac function during long-term hypoxia may be different from those which are responsible for early contractile failure in acute hypoxia and ischemia (1, 15, 40), highlighting the need for further studies.

Dihydropyridine and ryanodine receptors

It is widely accepted that (mainly) two groups of regulatory proteins control the entry of activator calcium to the myoplasm. Voltage-gated, dihydropyridine-sensitive (L-type) calcium channels (11, 22, 38) in the sarcolemma control the influx of extracellular calcium, and the ryanodine receptor (16, 25, 32, 38) controls the release of calcium stored in the sarcoplasmic reticulum. Thus, when $[Ca^{2+}]_o$ is elevated, the resulting increase in the calcium influx and intracellular calcium transient depends on the number of dihydropyridine-sensitive calcium channels and ryanodine receptors, and their functional state. We quantified both dihydropyridine and ryanodine receptors to determine whether their numbers change with exposure to long-term hypoxia, and to provide further insight into excitation-contraction coupling mechanisms in the late-term sheep fetus.

Our results indicate that the number of dihydropyridine receptors does not change in either ventricle following exposure to long-term hypoxia, but that the number of ryanodine receptors in the right ventricle, and the RyR: DHPR ratios in both ventricles increase. The B_{max} values for [^{3}H] (+)-PN200-110 were somewhat higher than those reported for other mammals (6, 22, 38), but the K_D values agreed well with previous

studies. The B_{max} and K_D values for [^{3}H] ryanodine in our study were similar to values reported previously (6, 25, 38). However, our B_{max} values were much lower than the 9.7 pmol/mg protein reported for the adult sheep left ventricle (16). In that study, Holmberg and Williams (16) used highly purified heavy SR vesicles collected from discontinuous sucrose density gradients, whereas we used a comparatively crude membrane preparation. They attributed the "unusually high level of ryanodine binding" to the high ionic strength of the 1 M KCl media used in their assay. We used 1 M NaCl which yielded a similarly high ionic strength. The differences in B_{max} values are probably related to the level of purity, and possibly to maturational changes in the SR.

Lew, Hryshko, and Bers (22) showed that the number of dihydropyridine receptors in the rabbit heart correlated closely with the number of functional calcium channels. If we assume a similar relationship in the sheep fetus, then our data suggest that the number of functional calcium channels in the heart is not altered by exposure to long-term hypoxia.

The increased number of ryanodine receptors in the right ventricle and RyR: DHPR ratios in both ventricles of hypoxic fetuses correlate with increased responsiveness to ryanodine (Figure 3). These two lines of evidence suggest that the SR and the contractile apparatus are both well developed in fetuses exposed to long-term hypoxia.

Conclusion

Long-term hypoxia decreased the inotropic response to extracellular calcium but did not change the number of sarcolemmal calcium channels. Dependence on ryanodine-sensitive calcium stores increased in parallel with a greater number of ryanodine receptors

in the right ventricle and larger RyR: DHPR ratios in both ventricles, indicating that the SR remained the chief source of activator calcium, and that hypoxia did not delay maturation of E-C coupling mechanisms. Together, these observations suggest that long-term hypoxia affects the functional state of key components of the contractile apparatus, including sarcolemmal calcium channels and ryanodine receptors. Further studies are needed to assess the calcium current, intracellular calcium transient, SR calcium loading and Ca^{2+}-ATPase activity, the amount of contractile protein, its calcium affinity, and ATPase activity.

Acknowledgments

The authors gratefully acknowledge Tim McNaughton, Al VanVarick, David Trydahl, and the White Mt. Research Station staff for their expert technical assistance. This work was supported by USPHS grants: HD22190 and HD03807.

References

1. Allen, D. G., and C. H. Orchard. Myocardial contractile function during ischemia and hypoxia. *Circ. Res.* 60: 153–168, 1987.

2. Alonso, J. G., T. Okai, L. D. Longo, and R. D. Gilbert. Cardiac function during long-term hypoxemia in fetal sheep. *Am. J. Physiol.* 257: H581–H589, 1989.

3. Anderson, P. A. W., A. Manring, and C. Crenshaw. Biophysics of the developing heart I. The force-interval relationship. *Am. J. Obstet. Gynecol.* 138: 33–43, 1980.

4. Anderson, P. A. W., A. P. Killam, R. D. Mainwaring, and A. E. Oakeley. In utero right ventricular output in the fetal lamb: the effect of heart rate. *J. Physiol. (Lond.)* 387: 297–316, 1987.

5. Anderson, P. A. W., K. L. Glick, A. Manring, and C. Crenshaw Jr. Developmental changes in cardiac contractility in fetal and postnatal sheep: in vitro and in vivo. *Am. J. Physiol.* 247 (*Heart Circ. Physiol.* 16): H371–H379, 1984.

6. Bers, D. M., and V. Stiffel. Ratio of ryanodine to dihydropyridine receptors in cardiac and skeletal muscle and implications for E-C coupling. *Am. J. Physiol.* 264 (*Cell Physiol.*): C1587–C1593, 1993.

7. Bers, D. M., J. H. B. Bridge, K. T. MacLeod. The mechanism of ryanodine action in rabbit ventricular muscle evaluated with Ca-selective microelectrodes and rapid cooling contractures. *Can. J. Physiol. Pharmacol.* 65: 610–618, 1987.

8. Bers, D. M., K. D. Philipson, and G. A. Langer. Cardiac contractility and sarcolemmal calcium binding in several cardiac muscle preparations. *Am. J. Physiol.* 240: H576–H583, 1981.

9. Bukauskas, F. Electrophysiology of the normal-to-hypoxic transition zone. *Circ. Res.* 51: 321-329, 1982.

10. Chin, T. K., W. F. Friedman, and T. S. Klitzner. Developmental changes in cardiac myocyte calcium regulation. *Circ. Res.* 67 (3): 574–579, 1990.

11. Cleeman, L., and M. Morad. Role of Ca^{2+} channel in cardiac excitation-contraction coupling in the rat: evidence from Ca^{2+} transients and contraction. *J. Physiol.* 432: 283-312, 1991.

12. Fabiato, A., and F. Fabiato. Calcium-induced release of calcium from sarcoplasmic reticulum of skinned cells from adult human, dog, cat, rabbit, rat and

frog hearts and from fetal and newborn rat ventricles. *Ann. N. Y. Acad. Sci.* 307: 491–522, 1978.

13. FABIATO, A. Time and calcium dependence of activation and inactivation of calcium-induced release of calcium from the sarcoplasmic reticulum of a skinned canine cardiac Purkinje cell. *J. Gen. Physiol.* 85: 247–290, 1985.

14. GLEED, R. D., E. R. POORE, J. P. FIGUEROA, AND P. W. NATHANIELSZ. Modification of maternal and fetal oxygenation with the use of tracheal gas infusion. *Am. J. Obstet Gynecol.* 155: 429–435, 1986.

15. HAJJAR, R. J., AND J. K. GWATHMEY. Direct evidence of changes in myofilament responsiveness to Ca^{2+} during hypoxia and reoxygenation in myocardium. *Am. J. Physiol.* 259 (Heart Circ. Physiol. 28): H784–H795, 1990.

16. HOLMBERG, S. R. M., AND A. J. WILLIAMS. The cardiac sarcoplasmic reticulum calcium-release channel: modulation of ryanodine binding and single-channel activity. *Biochim. Biophys. Acta* 1022: 187–193, 1990.

17. JARMAKANI, J. M., T. NAKANISHI, B. L. GEORGE, AND D. BERS. Effect of extracellular calcium on myocardial mechanical function in the neonatal rabbit. *Dev. Pharm. Ther.* 5: 1–13, 1982.

18. KAMITOMO, M., J. G. ALONSO, T. OKAI, L. D. LONGO, AND R. D. GILBERT. Effects of long-term, high-altitude hypoxemia on ovine fetal cardiac output and blood flow distribution. *Am. J. Obstet. Gynecol.* 169: 701–707, 1993.

19. KAMITOMO, M., L. D. LONGO, AND R. D. GILBERT. Cardiac function in fetal sheep during two weeks of hypoxemia. *Am. J. Physiol.* 266 (*Regulatory Integrative Comp. Physiol.* 35): R1778–R1785, 1994.

20. KAMITOMO, M., L. D. LONGO, AND R. D. GILBERT. Right and left ventricular function in fetal sheep exposed to long-term high-altitude hypoxemia. *Am. J. Physiol.* 262: H399–H405, 1992.

21. KENTISH, J. C. The effects of inorganic phosphate and creatine phosphate on force production in skinned muscles from rat ventricle. *J. Physiol.* 370: 585–604, 1986.

22. LEW, W. Y. W., L. V. HRYSHKO, AND D. M. BERS. Dihydropyridine receptors are primarily functional L-type calcium channels in rabbit ventricular myocytes. *Circ. Res.* 69: 1139–1145, 1991.

23. LEWARTOWSKI, B. AND B. PYTKOWSKI. Cellular mechanism of the relationship between myocardial force and frequency of contractions. *Prog. Biophys. Molec. Biol.* 50: 97–120, 1987.

24. Lewartowski, B., R. G. Hansford, G. A. Langer, and E. G. Lakata. Contraction and sarcoplasmic reticulum Ca^{2+} content in single myocytes of guinea pig heart: effect of ryanodine. *Am J. Physiol.* 259 (Heart Circ. Physiol. 28): H1222–H1229, 1990.

25. Lindsay, A. R. G., and A. J. Williams. Functional characterization of the ryanodine receptor purified from sheep cardiac muscle sarcoplasmic reticulum. *Biochim. Biophys. Acta* 1064: 89–102, 1991.

26. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193: 265-275, 1951.

27. Marban, E., and W. G. Wier. Ryanodine as a tool to determine the contributions of calcium entry and calcium release to the calcium transient and contraction of cardiac Purkinje fibers. *Circ. Res.* 56: 133–138, 1985.

28. Nakanishi, T., and J. M. Jarmakani. Developmental changes in myocardial mechanical function and subcellular organelles. *Pediatr. Res.* 10: 660-664, 1984.

29. Penefsky, Z. J., and M. Kahn. Mechanical and electrical effects of ryanodine on mammalian heart muscle. *Am. J. Physiol.* 218(6): 1682-1686, 1970.

30. Rueter, H. The dependence of the slow inward current on external calcium concentration in Purkinje fibers. *J. Physiol.* 192: 479-492, 1967.

31. Rousseau, E., and J. Pinkos. pH modulates conducting and gating behaviour of single calcium release channels. *Pflugers Arch.* 415: 645-647, 1990.

32. Rousseau, E. J., J. S. Smith, and G. Meissner. Ryanodine modifies conductance and gating behavior of single Ca^{2+} release channels. *Am. J. Physiol.* 253 (Cell Physiol. 22): C364–C368, 1987.

33. Smolich, J. J., A. M. Walker, G. R. Campbell, and T. M. Adamson. Left and right ventricular myocardial morphometry in fetal, neonatal, and adult sheep. *Am. J. Physiol.* 257: H1–H9, 1989.

34. Smolich, J. Morphology of the developing myocardium. In: *Research in Perinatal Medicine(V). Perinatal Development of the Heart and Lung*, edited by J. Lipshitz, J. Maloney, C. Nimrod, and G. Carson. Ithaca, N.Y.: Perinatology Press, 1987, 1–22.

35. Stern, M. D., H. S. Silverman, S. R. Houser, R. A. Josephson, M. C. Capogrossi, C. G. Nichols, W. J. Lederer, and E. G. Lakatta. Anoxic contractile failure in rat heart myocytes is caused by failure of intracellular calcium release due to alteration of the action potential. *Proc. Natl. Acad. Sci. USA* 85: 6954–6958, 1988.

36. Teitel, D. F., D. Sidi, T. Chin, C. Brett, M. A. Heymann, and A. M. Rudolph. Developmental changes in myocardial contractile reserve in the lamb. *Pediatr. Research* 19: 948-955, 1985.

37. Webster, K. A. and N. H. Bishopric. Molecular regulation of cardiac myocyte adaptations to chronic hypoxia. *J. Mol. Cell. Cardiol.* 24 (7): 741–752, 1992.

38. Wibo, M., G. Bravo, and T. Godfraind. Postnatal maturation of excitation-contraction coupling in rat ventricle in relation to the subcellular localization and surface density of 1,4-dihydropyridine and ryanodine receptors. *Circ. Res.* 68: 662–673, 1991.

39. Wier, W. G. Cytoplasmic [Ca^{2+}] in mammalian ventricle: dynamic control by cellular processes. *Annu. Rev. Physiol.* 52: 467–485, 1990.

40. Zhu, Y., and T. M. Nosek. Intracellular milieu changes associated with hypoxia impair sarcoplasmic reticulum Ca^{2+} transport in cardiac muscle. *Am. J. Physiol.* 261 (*Heart Circ. Physiol.* 30): H620–H626, 1991.

CHAPTER THREE

CARDIAC β-ADRENERGIC RECEPTOR FUNCTION IN FETAL SHEEP EXPOSED TO LONG-TERM HIGH-ALTITUDE HYPOXIA

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The experimental data in this paper were gathered and analyzed by the first author. Virginia Stiffel, Dr. Gilbert's technician, provided invaluable technical support, performing protein assays and recording ventricular and whole heart wet weights. The manuscript was written and revised by the first author, then reviewed and edited by Drs. Pearce, Longo, Gilbert, and Hessinger before being sent to the American Journal of Physiology.

Abstract

In fetal sheep, exposure to high-altitude (3,820 m) for ~110 days results in elevated arterial pressure, reduced cardiac output, and a dissociation between the chronotropic and inotropic responses to isoproterenol. In this study, we asked whether down-regulation of the myocardial β-adrenergic receptor/adenylate cyclase system may, in part, be responsible for the blunted inotropic response to isoproterenol. We recorded left and right ventricular wet weight, and measured the contractile response to increasing doses of isoproterenol and forskolin in papillary muscles from both ventricles. We estimated β-adrenergic receptor density (B_{max}) and ligand affinity (K_D) using [^{125}I] iodocyanopindolol (ICYP), and measured cyclic AMP levels before and after maximally stimulating doses of isoproterenol and forskolin. Left ventricular wet weight was unchanged, but right ventricular weight was ~20% lower than controls. At the highest concentration of isoproterenol (10 μM), maximum active tension was ~32% and ~20% lower than controls in hypoxic left and right ventricles, respectively. The contractile response to forskolin was severely attenuated in both hypoxic ventricles. B_{max} was unchanged in the left ventricle, but increased by 55% in the hypoxic right ventricle. K_D was not different from controls in either ventricle. Basal cAMP levels were not different from controls, but isoproterenol-stimulated and forskolin-stimulated cAMP levels were 1.4 to 2-fold higher than controls in both hypoxic ventricles. In summary, there was no ventricular hypertrophy, and the blunted contractile responses to isoproterenol and forskolin were not related to down-regulation of the β-adrenergic receptors or adenylate cyclase. The

results strongly suggest that hypoxia decreases A-kinase activity and/or the function of key target effector proteins.

Index terms: myocardial contractility, isoproterenol, forskolin, iodocyanopindolol

Introduction

During chronic hypoxia in adults (1, 14, 20, 21), cardiac output is reduced even though circulating levels of catecholamines remain elevated. Previous studies have suggested that increased parasympathetic activity (12), enhanced inactivation of catecholamines (20), and down-regulation of β-adrenergic receptors and adenylate cyclase (2, 15, 32) all play a role in the blunted response to β-stimulation. Several studies indicate that left and right ventricular β-receptors and adenylate cyclase activity are differentially regulated by chronic hypoxia. For example, in adult rats exposed to high-altitude, β-adrenergic receptor density decreased in the left ventricle after 3 wk (15) and remained decreased after 5 wk (32). In the right ventricle, β-receptor density was unchanged after 3 wk (15), but was decreased by ~50% after 5 wk (32). Basal and isoproterenol-stimulated adenylate cyclase activity were significantly reduced in the hypertrophied right ventricle (15), but not the left ventricle. Similar findings have been reported in newborn sheep made chronically hypoxic with right ventricular outflow tract obstruction, and a right to left shunt (2, 31, 9). Teitel et al. (31) showed a dissociation between the chronotropic and inotropic responses to elevated catecholamine levels. β-adrenergic receptor density and isoproterenol-stimulated adenylate cyclase activity (2) were decreased in the left ventricle, but were unchanged in the hypertrophied right ventricle. The authors suggested that in newborn lambs, hypoxia down-regulates β-receptors, while pressure overload up-regulates β-receptors, and causes ventricular hypertrophy. Using the same model, Doshi et al. (1991) (9) showed that the density and affinity of atrial β-adrenergic and muscarinic receptors were unchanged, suggesting that

during chronic hypoxia atrial and ventricular β-receptors are differentially regulated, resulting in different chronotropic and inotropic responses.

In fetal sheep exposed to long-term high-altitude hypoxia (16, 17, 18), baseline cardiac output was significantly reduced, owing mainly to a ~35% reduction in right ventricular output. When isoproterenol was infused in utero (18), arterial pressure fell significantly in both normoxic and hypoxic fetuses, and heart rate increased to a similar extent in both groups. Right ventricular output increased by ~35% in both groups, but left ventricular output increased by only ~15% in hypoxic fetuses compared to ~40% in normoxic controls, indicating a marked reduction in the left ventricular inotropic response to isoproterenol, and a dissociation between the chronotropic and inotropic responses.

In our model of chronic hypoxia, unlike the newborn lamb, plasma catecholamine levels were not elevated (13), and both left and right ventricles were exposed to the same levels of hypoxia and increased arterial pressure. However, the differences in fetal left and right ventricular afterload sensitivity and performance (24, 25), led us to hypothesize that hypoxia may differentially regulate ventricular β-receptors and adenylate cyclase in fetuses in a manner similar to that reported for the newborn lamb (2, 9). Such changes would have important implications for the transition from intrauterine to extrauterine life. In this study, we extended our previous observations in utero (16, 17, 18) to isolated papillary muscles from fetuses that were not surgically manipulated prior to study. We determined: (a) whether hypoxia resulted in left or right ventricular hypertrophy, (b) whether the inotropic response to isoproterenol and forskolin in isometrically contracting papillary muscle was altered by hypoxia, (c) whether the decreased inotropic response to

isoproterenol in the left ventricle in utero is related to down-regulation of the β-adrenergic receptor/adenylate cyclase system, (d) whether hypoxia differentially regulates left and right ventricular β-receptors, and (e) whether changes in β-receptor density and cyclic AMP levels might explain the decreased cardiac performance.

Methods

Seventy-eight time-dated pregnant ewes of a mixed western breed were obtained from Nebeker Ranch (Lancaster, CA) and randomly separated into control and long-term hypoxic groups. The control group (n=35) remained at Nebeker Ranch (altitude ~760 m) until 138–142 days gestation. At 30 days gestation, the long-term hypoxic group (n=43) was transported to Barcroft Laboratory, White Mountain Research Station (WMRS, Bishop, CA; altitude 3,820 m, barometric pressure ~480 Torr) where they remained until 138–142 days gestation. At both locations, the ewes were kept in a sheltered pen, and were provided with alfalfa pellets, mineral supplements, and clean water *ad libitum*. On the experimental day, the ewes were transported to our laboratory at Loma Linda University where they either underwent immediate study or, in the case of hypoxic ewes awaiting study, a nonocclusive tracheal catheter was surgically implanted so that N_2 gas could be administered to reestablish hypoxemia immediately after arrival at our laboratory. Experiments were scheduled so that fetuses were 142 d gestation on the experimental day. The ewes were sedated intravenously with thiamylal (10 mg/kg), intubated, and kept under deep surgical anesthesia (Halothane™ 5% in oxygen), while we delivered the fetuses through a midline laparotomy. The fetal hearts were removed via midline thoracotomy and placed

immediately in warm (39 °C), heparinized, low-calcium (0.2 mM Ca^{2+}) Tyrode solution continuously bubbled with 95% O_2–5% CO_2 for contractile studies or in ice-cold Tris buffer for the β-adrenergic receptor assay.

Whole heart and ventricular weights

The fetal heart was dissected free of the great vessels, and wet weights were recorded for the whole heart, and the left, and right ventricular free walls (Table 5). To determine percent dry weight, small sections of left and right ventricular free wall (1–3 g) were weighed, dried at ~250 °C for 5 days, then reweighed. The resulting dry weights were expressed as a percentage of the wet weight.

Contractile force

Tissue preparation. The detailed method of muscle preparation and stimulation has been previously described (6). Briefly, four thin papillary muscle strips (0.3-1.1 mm diameter) or trabeculae carnae (0.3–0.6 mm diameter) were excised from the fetal left (LV) and right (RV) ventricles. The muscles were stretched to their just-taut length, suspended in warm (39±0.1 °C), oxygenated (95% O_2-5% CO_2) Tyrode solution, and electrically stimulated at 1 Hz. Each contraction was recorded on an eight-channel polygraph (Gould Electronics, Cleveland, OH). Simultaneously, maximum active tension (T_{max}; g), and the maximum rates of rise ($+dT/dt_{max}$; $g \cdot sec^{-1}$) and fall ($-dT/dt_{max}$; $g \cdot sec^{-1}$) in tension were measured with a microcomputer (6, 8).

Isoproterenol-stimulated contractile response. After the muscles had equilibrated (~1 h), we recorded baseline control values, then measured the responses to cumulative doses of isoproterenol (10^{-10} to 10^{-5} M), a non-selective β-adrenergic receptor agonist. The stock

solution was taken directly from 5 ml ampuls of injectable isoproterenol hydrochloride (0.2 mg/ml Elkins-Sinn, Inc. Cherry Hills, NJ). After each addition of isoproterenol, we allowed the muscles to stabilize (~2 min), and at the plateau of each new steady-state recorded 20 contractions for later analysis. In preliminary experiments, increasing the maximum concentration of isoproterenol from 10^{-5} M to 10^{-4} M did not result in any additional increase in contractility. Contractile force consistently reached a new plateau within ~60–90 sec after adding isoproterenol, and remained stable for up to 15 minutes in both normoxic (n=10) and hypoxic (n=10) fetuses. Some muscle strips developed rapid (~3 Hz), spontaneous, phasic contractions of reduced amplitude when isoproterenol concentration was increased above ~0.3 μM. Those muscles were excluded from the study. All experiments were conducted in the dark to protect isoproterenol from photolytic degradation.

In preliminary experiments, 10 μM propranolol was added to the bath medium after the muscles had equilibrated to determine whether endogenous catecholamines affected baseline T_{max} and $\pm dT/dt_{max}$. After a 20 minute incubation, there was no significant change in baseline values in both normoxic and hypoxic fetuses. However, 10 μM propranolol completely blocked the contractile response to isoproterenol in both groups.

Forskolin-stimulated contractile response. In a second set of muscles, we measured the responses to cumulative doses of forskolin (10^{-8} to 10^{-5} M), a direct activator of adenylate cyclase. Forskolin (5 mg vial, Calbiochem) was dissolved in 180 μl dimethyl sulfoxide (DMSO) and diluted with distilled water to make a 2.5 mM stock solution in 6% DMSO. After each addition of forskolin, we allowed the muscles to stabilize (~15 minutes), and at the plateau of each new steady-state recorded 20 contractions for later

analysis. In preliminary experiments, increasing the maximum forskolin concentration from 10 μM to 200 μM did not result in any additional increase in contractile force.

β-adrenergic receptor assay

Approximately 1 gram each of the left and right ventricular free walls was placed in ice-cold buffer containing 20 mM Tris (Tris[hydroxymethyl]aminomethane), 250 mM sucrose, and 1 mM dithiothreitol (DTT), pH 7.4, then homogenized with a Polytron (Brinkman Instruments, Westbury, NY). The homogenate was spun at 110,000g (50,000 rpm) for 45 minutes at 4 °C in an ultracentrifuge equipped with a Ti-50 rotor (Beckman Instruments model L3-50). The pellet fraction was resuspended in buffer containing 50 mM Tris, pH 7.4, then stored at -70 °C in sealed cryogenic vials. Samples were stored for no more than two months before assay.

On the assay day, 5 ml of frozen sample was resuspended in 10 ml ice-cold 50 mM Tris buffer, then homogenized thoroughly with a glass mortar and pestle before being passed through two layers of gauze to remove cellular debris. The homogenate was diluted with an equal volume of assay buffer (2X stock) to a final protein concentration of 1–2 mg/ml. The resulting membrane suspension contained 50 mM Tris, 4 mM $MgCl_2$, and 1 mM ascorbic acid, pH 7.4, to which was added a cocktail of protease inhibitors in a final concentration of: 76.8 nM aprotinin, 0.83 mM benzamidine, 1 mM iodoacetamide, 1.1 μM leupeptin, 0.7 μM pepstatin-A, and 0.23 mM PMSF (phenylmethylsulfonyl fluoride). [^{125}I] (-)Iodocyanopindolol (ICYP) (New England Nuclear) was used to estimate the density of left and right ventricular β-adrenergic receptors. The assay was performed in triplicate in tubes containing 240 μl of diluted membranes and 10 μl of increasing concentrations of the radioligand (1–1,000 pM).

Non-specific binding was determined by adding 10 µl of (-)isoproterenol (200 µM stock) to one set of assay tubes. Following a 1 hr incubation in the dark at 30 °C, the membranes were collected by vacuum filtration on glass fiber filter circles G4 (Fisher Scientific), and rinsed three times with 5 ml of ice-cold 50 mM Tris, and 4 mM $MgCl_2$, pH 7.4, to remove unbound radiolabel. When the filters were dry, they were placed in 12x75 polyethylene tubes, and the radioactivity was measured in a gamma counter (Packard Autogamma model 5650). The remaining membrane suspension was analyzed for protein by the Lowry method(19).

Cyclic AMP determination

Isoproterenol-stimulated cAMP production. After a 1 h equilibration in Tyrode solution (see above), actively contracting papillary muscles or trabeculae carnae from normoxic (n=35) and hypoxic fetuses (n=43) were treated with a single bolus of 2 µM isoproterenol hydrochloride. When the contractile response reached its maximum (~45–60 sec), the muscles were rapidly frozen by immersion in liquid nitrogen. Untreated left and right ventricular muscle strips from each fetus were also frozen as controls. The frozen muscles were placed in labeled aluminum foil pouches and stored at -70 °C in sealed cryogenic vials until assay. In 10 fetuses from each group, we recorded 20 contractions at baseline and after stimulation with isoproterenol in an attempt to correlate contractility with cAMP levels.

Forskolin-stimulated cAMP production. After the dose-response curve to forskolin had been constructed (see above), the muscles were rapidly frozen by immersion in liquid nitrogen, then stored at -70 °C in sealed cryogenic vials for later determination of steady-state cyclic AMP levels. In separate experiments, a second group of muscles were

treated with a single bolus of 10 μM forskolin, the maximum concentration used in the dose-response study. When the contractile response reached a new plateau (~10 min), the muscles were rapidly frozen by immersion in liquid nitrogen and stored as described above.

Cyclic AMP assay. On the first assay day, the frozen muscle strips were transferred directly from the -70 °C freezer to a Dewar flask filled with liquid nitrogen. Individual muscle strips were removed from the liquid nitrogen, then immediately homogenized in 1 ml ice-cold 6% trichloroacetic acid (TCA) with a glass mortar and pestle. The mortar and pestles were rinsed twice with 1 ml ice-cold 6% TCA, and the pooled 3 ml volume was centrifuged at 2,000g for 15 minutes at 4 °C. The pellet fraction was used for protein determination by the Lowry method (19). The supernatant was washed four times with 5 ml water-saturated diethyl ether, then lyophilized (Savant Speedvac Condenser model SVC-200H) overnight at 60 °C. On assay day-2, the dried extract was resuspended in 1 ml of the 0.05 M acetate buffer provided in Amersham's cAMP [^{125}I] assay system (dual range) kit. Tissue levels of cAMP were determined using the non-acetylation assay according to the method described in the Amersham kit. A total of 332 muscle strips from 35 normoxic and 43 hypoxic animals were processed.

Data Analysis

Whole heart and ventricular weights. Individual fetal left and right ventricular data were pooled to calculate normoxic and hypoxic group means.

Contractile force. Baseline values recorded at the end of the 1 h equilibration were time-averaged and designated the control value (100%). For each fetus, the 20 contractions recorded at each concentration of isoproterenol and forskolin were averaged,

expressed as a percentage of the baseline control value, then plotted against log [agonist]. The data were fit to Hill curves using the non-linear regression analysis algorithms in GraphPAD Prism® (GraphPAD™ Software, San Diego, CA). Pooled data from normoxic and hypoxic groups were used to fit the curves displayed in Figs. 1 and 3, and to determine the EC_{50} values in Tables 3 and 4.

The 20 contractions recorded after stimulating the muscles with 2 μM isoproterenol were averaged and expressed as a percent of the baseline control value. The resulting individual fetal data were pooled to calculate normoxic and hypoxic group means.

β-*adrenergic receptor and cAMP assays.* The radioactivity measured in triplicate samples was averaged to produce a single value for each concentration of the radioligand. Raw counts were converted to femtomoles of β-receptor or cAMP per milligram of protein, and, for [^{125}I] ICYP, the resulting data were fit to a rectangular hyperbola using the non-linear regression analysis algorithms in Prism®. Pooled data from normoxic and hypoxic fetuses were used to fit the curves, and to calculate B_{max} and K_D values, which are reported in Fig. 9 and Table 9. Similar results were obtained whether group data were curve fit or if curve fit parameters from individual fetuses were averaged.

Statistics

The dose-response curves for isoproterenol and forskolin, and the cAMP data were analyzed in SPSS® (SPSS Inc., Chicago, IL) using doubly multivariate repeated measures analysis of variance for a split-plot design. For all three analyses, ventricle was a within-subjects factor with 2 levels (LV and RV), and oxygen was the between-subjects factor with 2 levels (hypoxia or normoxia). In their respective analyses, isoproterenol and

forskolin concentrations were within-subjects factors with 21 and 6 levels, respectively. T_{max}, and $\pm dT/dt$ were the measures. In the analysis of the cAMP data, the drug used to stimulate cAMP production was a within-subjects factor with three levels: baseline control (no drug), 2 μM isoproterenol, and 10 μM forskolin. Because the raw cAMP data were not normally distributed, $\log_{10}$-transformed data were analyzed. Eight hypoxic and three normoxic fetuses were excluded from the repeated measures analysis because of missing data. Student's *t*-test was used to compare normoxic and hypoxic group means for whole heart and ventricular weights, the EC_{50} values for isoproterenol and forskolin, the bolus of 2 μM isoproterenol, and the B_{max} and K_D values for [^{125}I] ICYP. For all comparisons, statistical significance was set at $P<0.05$. Results were expressed as means ± SE.

Results

Whole heart and ventricular wet weights

Whole heart and left ventricular wet weights were unchanged by long-term hypoxia; right ventricular wet weight was decreased by ~20% in hypoxic fetuses (Table 5). Dry weight was slightly increased in the left ventricle but not in the right ventricle of hypoxic fetuses.

Contractile response to isoproterenol

At the end of the 1 h equilibration period, T_{max}, $\pm dT/dt_{max}$, and the time course of contraction were similar in normoxic and hypoxic fetuses. Isoproterenol increased contractile force in a dose-dependent manner in both normoxic and hypoxic fetuses (Fig. 6). In normoxic fetuses, T_{max} and $+dT/dt_{max}$ increased ~3 to 4-fold in both left and right

TABLE 5. Ventricular and whole heart weights.

Experimental Group	Left Ventricle	Right Ventricle	Whole Heart
Normoxic			
Wet weight	9.68 ± 0.55	8.28 ± 0.48	23.95 ± 1.32
% Dry weight	16.94 ± 0.54	17.14 ± 0.68	———————
Hypoxic			
Wet weight	9.37 ± 0.64	6.65 ± 0.34*	20.48 ± 1.23
% Dry weight	18.76 ± 0.58*	18.67 ± 0.55	———————

Whole heart and ventricular wet weights, and dry weight expressed as a percentage of wet weight in normoxic (N=21) and hypoxic fetuses (N=19). Values are means±SE given in grams. *P <0.05 compared with normoxic.

ventricles, and $-dT/dt_{max}$ increased ~7-fold in the left ventricle versus ~3-fold in the right ventricle (Fig. 6 and Table 6). The hypoxic left and right ventricles were less responsive to isoproterenol. In the hypoxic left ventricle, the maximum responses were reduced by ~32%, ~28%, and ~66% for T_{max}, $+dT/dt_{max}$, and $-dT/dt_{max}$, respectively (Fig. 6 and Table 6). In the hypoxic right ventricle, all three measures of contractility were decreased by ~20%.

The isoproterenol dose-response curves in hypoxic fetuses were left-shifted when compared to the corresponding curves for normoxic fetuses (Fig. 6). As a result, the concentration of isoproterenol at which the contractile responses were half-maximal (EC_{50}) was significantly lower in hypoxic fetuses (Table 7). In the hypoxic left ventricle, EC_{50} values were ~18 times lower for T_{max} and $+dT/dt_{max}$, and ~55 times lower for $-dT/dt_{max}$ than in the normoxic left ventricle. In the hypoxic right ventricle, the EC_{50} values for all three measures of contractility were ~3 times lower than in the normoxic right ventricle. The normoxic left ventricle was significantly less sensitive to isoproterenol than the normoxic right ventricle, as indicated by the ~6 to 18-fold difference in their EC_{50} values (Table 7). In contrast, the hypoxic left and right ventricles did not differ in their sensitivities to isoproterenol, suggesting that hypoxia alters the normal differential sensitivity to β-adrenergic agonists.

When a single bolus of 2 μM isoproterenol was used to stimulate the muscles (for later cAMP determinations), the contractile response was significantly reduced in both the hypoxic left and right ventricles (Fig. 7). T_{max} and $\pm dT/dt_{max}$ were reduced by ~30% and ~48%, respectively, in the hypoxic left ventricle. Similarly, T_{max} and $+dT/dt_{max}$ were reduced by ~30% in the hypoxic right ventricle but $-dT/dt_{max}$ was unchanged. Together,

4

Figure 6. The contractile response to increasing concentrations of soproterenol in papillary muscles from the left (Panels A, B, C) and right (Panels D, E, F) ventricles of normoxic (■) and chronically hypoxic (□) fetuses. Maximum active tension (T_{max}), maximum rate of rise in tension ($+dT/dt_{max}$), and maximum rate of relaxation ($-dT/dt_{max}$). N=10 fetuses in each group.

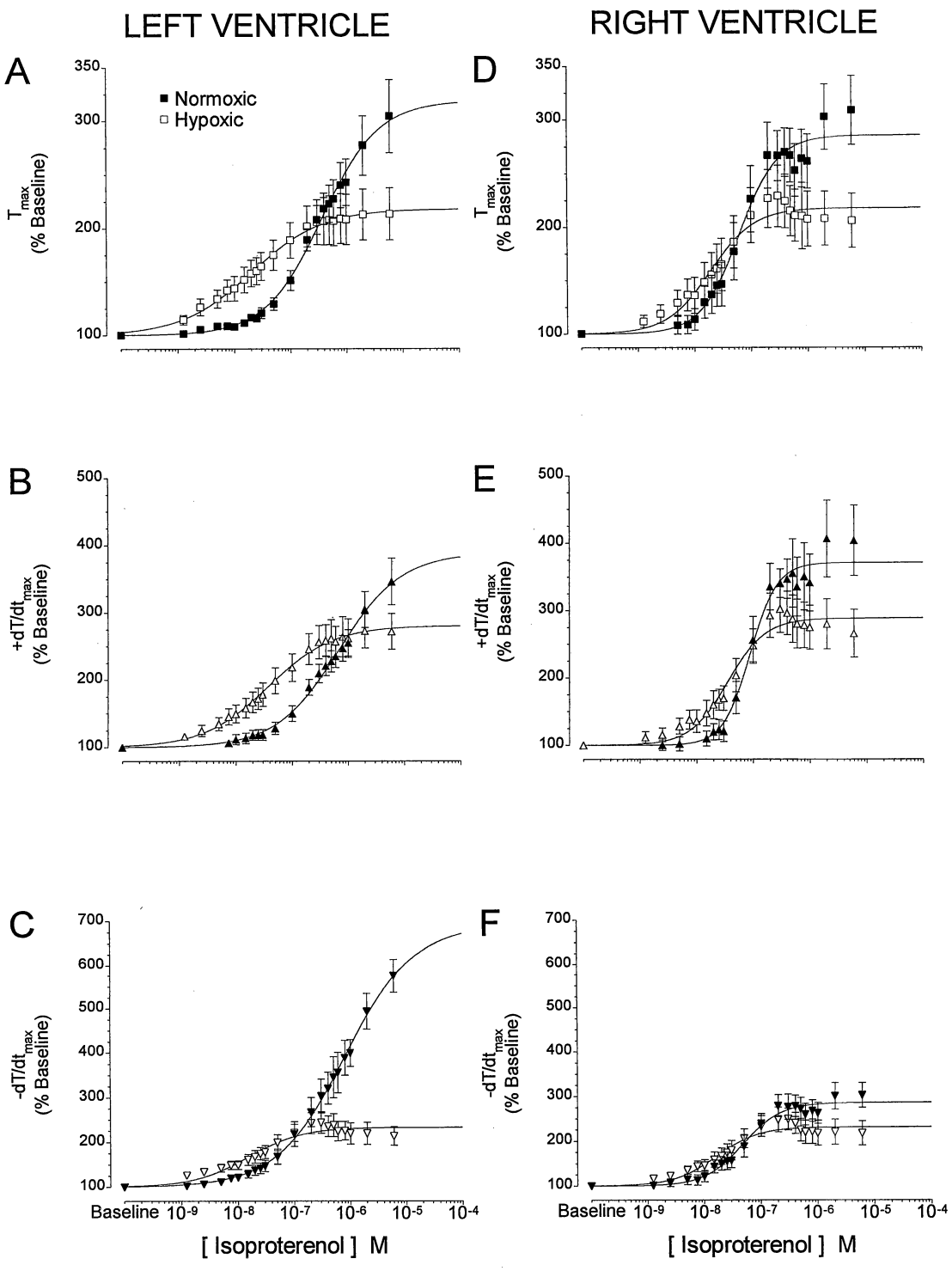

LEFT VENTRICLE
RIGHT VENTRICLE
A
B
C
D
E
F
■ Normoxic
□ Hypoxic
T_{max} (% Baseline)
+dT/dt$_{max}$ (% Baseline)
-dT/dt$_{max}$ (% Baseline)
350
300
200
100
500
400
700
600
Baseline 10^{-9} 10^{-8} 10^{-7} 10^{-6} 10^{-5} 10^{-4}
[Isoproterenol] M

TABLE 6. Maximum values for isoproterenol dose-response curves.

	LEFT VENTRICLE			RIGHT VENTRICLE		
Variable	Normoxic	Hypoxic	%Δ	Normoxic	Hypoxic	%Δ
Tmax	319 ± 7	218 ± 2*	-31.7	285 ± 6	218 ± 4*	-23.5
+dT/dt	388 ± 12	280 ± 3*	-27.8	371 ± 8	288 ± 6*	-22.4
−dT/dt	692 ± 19	233 ± 6*	-66.3	286 ± 6‡	231 ± 5*	-19.2

The top-plateau values from the isoproterenol dose-response curves in Figure 6. The data represent the maximum responses from individual fetal curves. Values are group means±SE; N=10 in each group. $P < 0.0001$ compared with normoxic controls (*) and left ventricle (‡).

TABLE 7. EC_{50} values for isoproterenol dose-response curves.

	LEFT VENTRICLE		RIGHT VENTRICLE	
Variable	Normoxic	Hypoxic	Normoxic	Hypoxic
Tmax	371 ± 37	20 ± 1*	62 ± 8‡	19 ± 2*
+dT/dt	679 ± 88	38 ± 3*	89 ± 9‡	36 ± 4*
−dT/dt	832 ± 86	15 ± 3*	47 ± 6‡	16 ± 2*

Isoproterenol concentration (in nM) at which the increase in contractile activity was half-maximal (EC_{50}) from the curve fits in Figure 6. Values are means±SE; N=10 in each group. *P<0.01 compared with normoxic controls. ‡P<0.01 compared with left ventricle.

these two experiments indicate that the positive inotropic response to isoproterenol is decreased in both hypoxic ventricles, suggesting altered β-adrenergic receptor function.

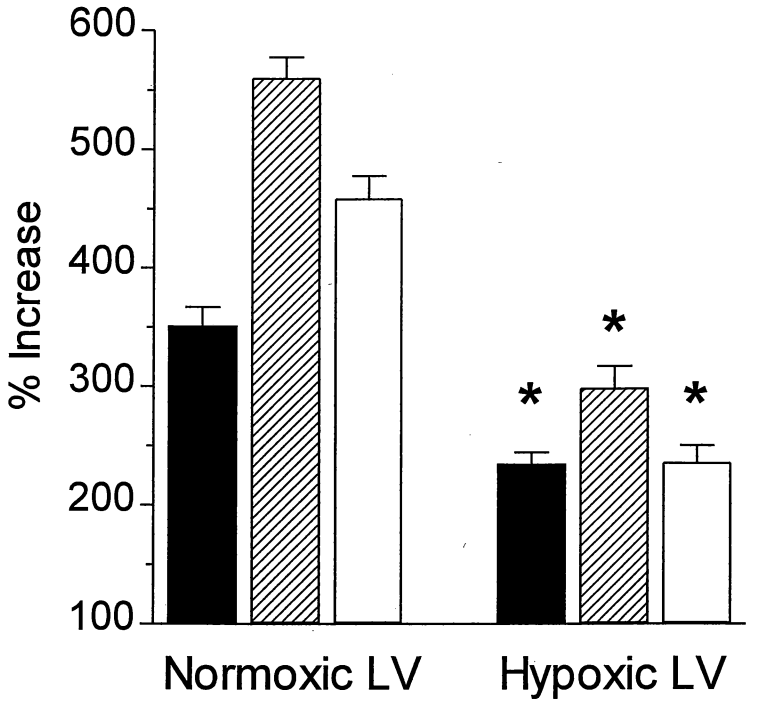
Contractile response to forskolin

Forskolin increased contractility in a dose-dependent manner in normoxic fetuses (Fig. 8). In both the left and right ventricles, T_{max} and $\pm dT/dt_{max}$ increased ~2 to 4-fold over baseline values. The left ventricle was more sensitive to forskolin than the right ventricle as indicated by the lower EC_{50} values in Table 8. In hypoxic fetuses, the contractile response to forskolin was severely blunted, even when the forskolin concentration was increased to 200 μM (data not shown). Because the response was not sigmoidal, meaningful maximum response and EC_{50} values could not be determined.

Quantification of β-adrenergic receptors

For both normoxic (n=18) and hypoxic (n=16) fetuses, [^{125}I] (-)Iodocyanopindolol (ICYP) bound to a single class of high-affinity binding sites (data not shown). The resulting B_{max} and K_D values are shown in Table 9. In normoxic fetuses, β-adrenergic receptor density (B_{max}) was ~33% higher in the left ventricle than in the right ventricle. In hypoxic fetuses, there was no difference in β-receptor density between left and right ventricles. Exposure to long-term hypoxia did not change β-receptor density in the left ventricle. However, there was a ~55% increase in β-receptor density in the hypoxic right ventricle, indicating hypoxic up-regulation of right ventricular β-receptors. There was no difference in ligand affinity (K_D) between left and right ventricles, or between normoxic and hypoxic fetuses.

Figure 7. Contractile response to a bolus of 2 μM isoproterenol in papillary muscles from the left (Panel A) and right (Panel B) ventricles of normoxic (N=10) and chronically hypoxic (N=10) fetuses. *P<0.01 compared to normoxic controls.


600
500
400
300
200
100
% Increase
*
*
*
Normoxic LV
Hypoxic LV

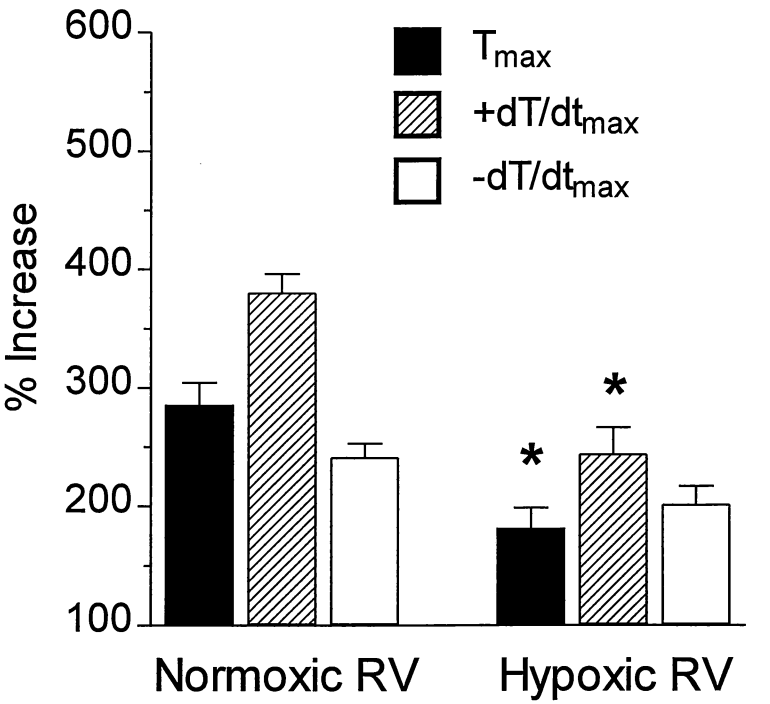

600
500
400
300
200
100
% Increase
T_{max}
$+dT/dt_{max}$
$-dT/dt_{max}$
*
*
Normoxic RV
Hypoxic RV

Figure 8. The contractile response to increasing concentrations of forskolin in papillary muscles from the left (Panels A, B, C) and right Panels D, E, F) ventricles of normoxic (■) and chronically hypoxic (□) fetuses. N=8 fetuses in each group. Maximum active tension (T_{max}), maximum rate of rise in tension ($+dT/dt_{max}$), and maximum rate of relaxation ($-dT/dt_{max}$).

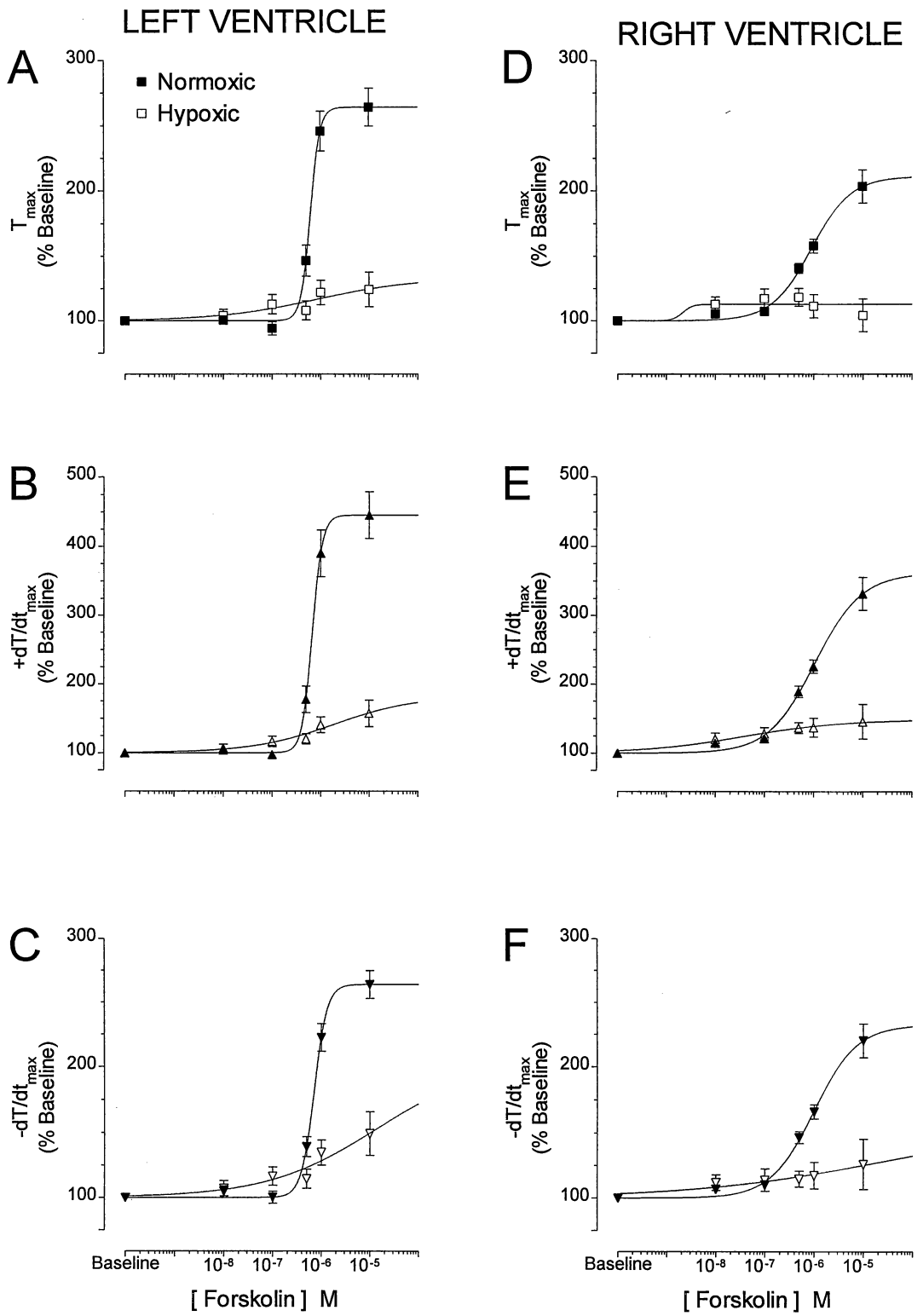

LEFT VENTRICLE
RIGHT VENTRICLE
A
B
C
D
E
F
■ Normoxic
□ Hypoxic
T_{max} (% Baseline)
$+dT/dt_{max}$ (% Baseline)
$-dT/dt_{max}$ (% Baseline)
300
200
100
500
400
Baseline
10^{-8}
10^{-7}
10^{-6}
10^{-5}
[Forskolin] M

TABLE 8. EC_{50} values for forskolin dose-response curves in normoxic fetuses.

Variable	Left Ventricle	Right Ventricle
Tmax	0.62 ± 0.02	0.893 ± 0.07*
+dT/dt	0.67 ± 0.01	1.067 ± 0.13*
−dT/dt	0.72 ± 0.02	0.983 ± 0.09*

Forskolin concentration (in μM) at which the increase in contractile activity in normoxic fetuses was half-maximal (EC_{50}) from the curve fits in Figure 3. Because the Forskolin response in hypoxic fetuses was greatly attenuated and was not sigmoidal, meaningful EC_{50} values could not be determined. Values are means±SE; N=8. *P<0.01 compared with left ventricle.

Cyclic AMP levels

Fig. 10 shows the results from the cAMP assays. Basal unstimulated cAMP levels were not significantly different in normoxic and hypoxic fetuses, or between left and right ventricles. When 2 μM isoproterenol was used to activate β-receptor-coupled adenylate cyclase, cAMP concentration increased significantly in both normoxic and hypoxic fetuses. There were no significant differences between left and right ventricles in either group. However, the increases in cAMP were ~1.8 fold and ~1.4-fold higher in hypoxic left and right ventricles, respectively, than in the corresponding normoxic ventricle (Fig. 5). When adenylate cyclase was directly activated with 10 μM forskolin, a similar pattern emerged. Forskolin-stimulated cyclic AMP levels were ~5-fold higher than basal unstimulated levels in hypoxic fetuses, but only ~3-fold higher than basal unstimulated levels in normoxic fetuses (Fig. 5). There were no significant differences between left and right ventricles in either group.

Discussion

Whole heart and ventricular weights

Several investigators have reported right ventricular hypertrophy following exposure to chronic hypoxia in adult (15, 32) and newborn (2) mammals. We found that right ventricular hypertrophy did not occur in fetal sheep exposed to long-term hypoxia. Instead, the mass of the right ventricular free wall decreased by ~20% without any compensatory change in the left ventricle. The decrease in right ventricular free wall mass may, in part, help to explain the reduced right ventricular performance previously reported by our laboratory (16, 17, 18). Differences in the period of hypoxic exposure, and

Figure 9. β-adrenergic receptor density in partially purified membranes from normoxic (N=18) and hypoxic (N=16) fetuses. $P<0.0001$ compared with normoxic (*); compared with left ventricle ($^{+}$).

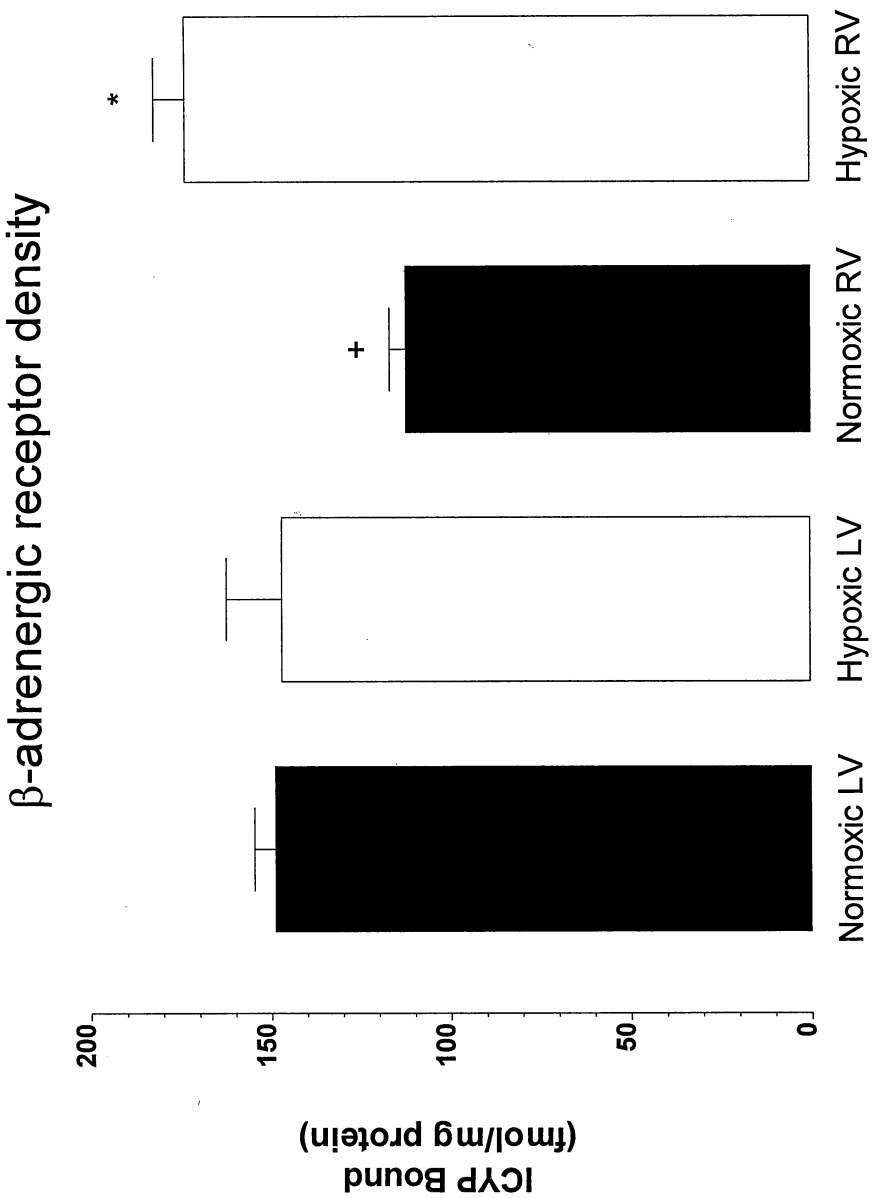
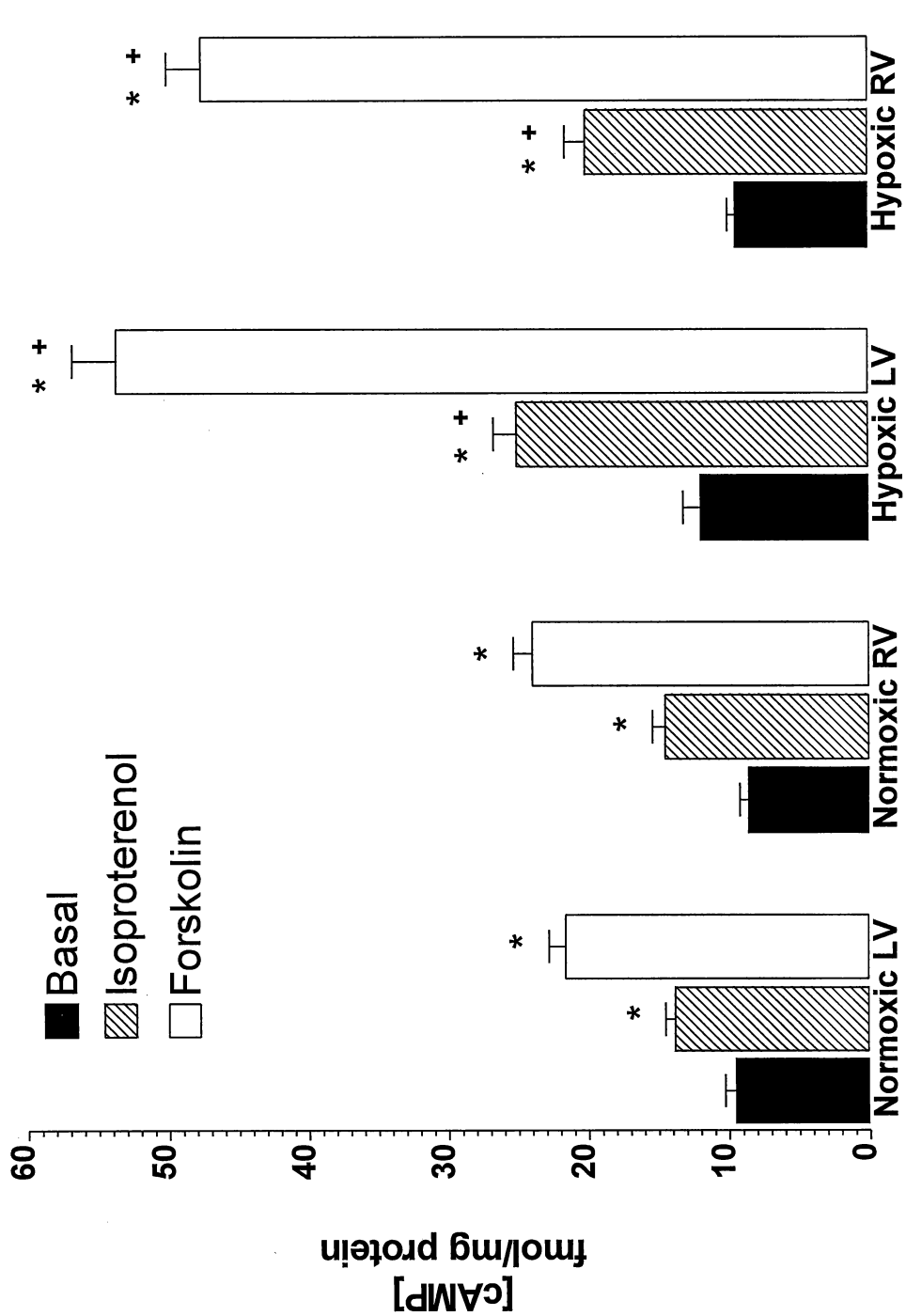

β-adrenergic receptor density
ICYP Bound
(fmol/mg protein)
200
150
100
50
0
*
+
Normoxic LV
Hypoxic LV
Normoxic RV
Hypoxic RV

TABLE 9. Left and right ventricular β-adrenergic receptor density (B_{max}) and affinity (K_D).

	LEFT VENTRICLE		RIGHT VENTRICLE	
	B_{max}	K_D	B_{max}	K_D
Normoxic:	149 ± 6	287 ± 30	112 ± 4‡	266 ± 29
Hypoxic:	147 ± 15	354 ± 89	174 ± 8*	366 ± 42

β-adrenergic receptor density, B_{MAX} (fmol·mg^{-1} protein), and ligand affinity, K_D (pM), for [^{125}I]-iodocyanopindolol in membranes from normoxic (N=18) and hypoxic (N=16) fetal hearts. Values are means ± SE. *P <0.0001 compared with normoxic. ‡P <0.0001 compared with left ventricle.

Figure 10. Cyclic AMP levels in normoxic (N=32) and hypoxic (N=35) fetuses before (Baseline) and after stimulation with 2 μM isoproterenol or 10 μM forskolin. *P<0.0001 compared with baseline; ⁺P<0.0001 compared with normoxic fetuses.


Basal and Stimulated Intracellular cAMP Levels
Basal
Isoproterenol
Forskolin
[cAMP]
fmol/mg protein
60
50
40
30
20
10
0
Normoxic LV
Normoxic RV
Hypoxic LV
Hypoxic RV
*
*
*
*
*
*
*
*
+
+
+
+

the morphology and composition of the fetal myocardium vs. the newborn myocardium may account for the differences between our results and those of Bernstein et al. (2). In their study, newborn lambs were exposed to 2 wk of hypoxia beginning during the first and second weeks of postnatal life; our fetuses were exposed to hypoxia during days 30–142 of gestation, a period during which myocardial cells are actively dividing (5, 29, 30). During the last week of fetal life and the first week of neonatal life, mitotic activity tapers off and myocyte hypertrophy, especially in the left ventricle, becomes the dominant mechanism for myocardial growth (5, 29, 30). In rats, chronic hypoxia during fetal life results in marked hyperplasia and delayed transition from hyperplastic to hypertrophic growth in the right ventricle but not in the left ventricle (22). Whether a similar mechanism may be at work in chronically hypoxic fetal sheep is not known.

Contractile responses to isoproterenol and forskolin

In the intact heart, changes in cardiac performance may be related to changes in preload, afterload, heart rate, or inotropic state. By studying papillary muscle that is electronically paced and contracting isometrically, we can directly assess changes in inotropy without the confounding influences of cardiovascular reflexes. The inotropic responses to β-adrenergic receptor agonists are mediated by a cascade of enzymatic reactions which culminate in the phosphorylation of key proteins that are important for excitation-contraction coupling. Sarcolemmal β-receptors are coupled to adenylate cyclase by the guanine nucleotide proteins (G-proteins) G_s and G_i. Activation of the β-receptor–G-protein–adenylate cyclase complex results in increased cAMP production, and increased cAMP-dependent protein kinase (A-kinase) activity. A-kinases phosphorylate key

Ca^{2+} channels in the sarcolemma and sarcoplasmic reticulum, phospholamban, troponin-I, and C-protein. Phosphorylation of calcium channels in the sarcolemma and sarcoplasmic reticulum increase both the influx of extracellular calcium and the release of calcium stored in the sarcoplasmic reticulum. The resulting increased delivery of calcium to the myofilaments is chiefly responsible for the increase in contractile force. Phosphorylation of phospholamban increases the rate of calcium reuptake by the sarcoplasmic reticulum Ca^{2+}-AT Pase pump, while the phosphorylation of troponin-I and C-protein decreases myofilament sensitivity for myoplasmic calcium. The increased SR calcium uptake and decreased myofilament sensitivity for calcium are chiefly responsible for the more rapid decline in the calcium transient and the increased rate of relaxation. Changes in responsiveness and sensitivity to β-agonists could, in theory, occur as a result of changes at any level in the signal transduction pathway beginning at the β-receptor and ending at the effector proteins. In this study, we explored the inotropic responsiveness and sensitivity to a β-receptor agonist, isoproterenol, and a direct activator of adenylate cyclase, forskolin. In addition, we measured the density of β-adrenergic receptors, and determined the levels of cAMP following stimulation with isoproterenol and forskolin.

In adult and newborn mammals, several investigators have shown that prolonged exposure to elevated catecholamine levels during acute (26) and chronic hypoxia (1, 2, 12, 15, 18, 21), heart failure (4, 11), and pressure overload (2, 28, 32), results in a marked decrease in myocardial responsiveness to β-stimulation. This may, in part, be explained by down-regulation of surface β-receptors (2, 9, 15, 32) and decreased adenylate cyclase activity (2, 15, 32, 33). Recently, we reported (18) a marked decrease in the left

ventricular inotropic response to isoproterenol in fetal sheep exposed to long-term high-altitude hypoxia. In this study, we hypothesized that the reduced inotropic response to isoproterenol in vivo may be due to down-regulation of the β-adrenergic receptor/adenylate cyclase system.

There were several important findings in this study. First, isoproterenol was a potent positive inotropic agent in both normoxic and hypoxic fetuses. However, in the hypoxic left and right ventricles, the inotropic response to isoproterenol was markedly attenuated (Figs. 6 & 7 and Table 6), but sensitivity was greatly increased (Table 7). The changes were more pronounced in the hypoxic left ventricle, particularly for $-dT/dt_{max}$: inotropic responsiveness decreased by 66%, but sensitivity increased 55-fold. These results agree with the decreased left ventricular inotropic response to isoproterenol in vivo (18). However, unlike the results in this study, the right ventricular response in vivo was not different from normoxic controls. This inconsistency may be the result of a key difference in methodology. In this study, afterload remained constant (isometric contraction), but arterial pressure fell significantly during the isoproterenol infusion in vivo, thereby, reducing afterload and improving stroke volume. Because the right ventricle is quite sensitive to changes in afterload (17, 24, 25), the reduction in arterial pressure may have been sufficient to compensate for an underlying decrease in responsiveness to isoproterenol.

The pattern of the inotropic response to isoproterenol in hypoxic fetuses is similar to the inotropic response to extracellular calcium previously reported (6). In both studies, inotropic responsiveness was markedly attenuated, but sensitivity was greatly increased. In both studies, the changes were of a similar magnitude, and were more pronounced in the

left ventricle, particularly for $-dT/dt_{max}$ (see Table 1 and Figs. 1&2 in ref 6). Because the inotropic response to isoproterenol depends on calcium delivery, reuptake, and changes in myofilament sensitivity to calcium, we hypothesize that the changes in the inotropic response to calcium and isoproterenol in hypoxic fetuses are linked by a common, as yet unexplored, mechanism.

A second finding was that forskolin had a potent positive inotropic effect on the normoxic fetal heart. Overall, the magnitude of the contractile response was similar to that observed for isoproterenol. However, isoproterenol was a potent inotrope when maximal inotropic response and drug sensitivity were compared (see Figs. 1–3 and Tables 2–4). In addition, the response to isoproterenol reached a new plateau within 60–90 sec, while the response to forskolin required ~10 min to reach a new steady-state. The difference in the rate of activation may, in part, be explained by the fact that isoproterenol binds to surface receptors while forskolin has to diffuse across the cell membrane to activate adenylate cyclase. These results agree with previous observations on the relative potencies of isoproterenol, isoprenaline, and forskolin in human (3) and rat (10) hearts.

In hypoxic fetuses, the inotropic response to forskolin was even more severely attenuated than the inotropic response to isoproterenol (Fig. 8). In several hypoxic fetuses, contractility decreased below baseline values (Fig. 8), suggesting that forskolin may have been toxic, especially in the right ventricle. Alternatively, forskolin activates all adenylate cyclases, while isoproterenol activates only the subset of adenylate cyclases that are coupled to the β-adrenergic receptor and to the contractile response (3, 10, 27). Thus, the contractile responses to isoproterenol and forskolin could, in theory, differ considerably depending on the activity of A-kinase, and the phosphorylation states of

membrane and myofilament effector proteins. In fact, in the isolated perfused rat heart, England and Shahid, 1987 (10) showed that isoprenaline was a more potent inotrope than forskolin on a molar basis, and that A-kinase activity, phosphorylase *a* content, and the levels of phosphorylated troponin-I and C-protein were much higher after stimulation with isoprenaline than with forskolin, even though forskolin-stimulated cAMP levels were much higher than isoprenaline-stimulated cAMP levels. They concluded that much of the cAMP produced by stimulation with forskolin was unavailable to the A-kinases that are involved in the contractile response because of compartmentation. It is not clear why these differences may be more apparent in hypoxic fetuses than in normoxic fetuses. We speculate that A-kinase activity and or the phosphorylation states of target effector proteins, and hence their physiological activity, may be reduced following exposure to long-term high-altitude hypoxia.

β-adrenergic receptor density and agonist affinity

The third major finding in this study was that long-term high-altitude hypoxia did not result in down-regulation of ovine fetal myocardial β-adrenergic receptors. Instead, there was a 55% increase in right ventricular β-adrenergic receptor density in hypoxic fetuses, but no change in the left ventricle. In addition, there were no changes in the affinity for [^{125}I]-iodocyanopindolol in either ventricle. In normoxic fetuses, the B_{max} for [^{125}I] ICYP in our study was higher than that previously reported for late-term fetuses (7, 23), but lower than that reported for normoxic newborn lambs (2). In our study, normoxic fetal left and right ventricular β-receptor density were ~45% and ~55%, respectively, of that reported for the 2–3 wk old newborn, suggesting that there is an age-related increase in β-receptor density during the early neonatal period. Hypoxic fetal

left and right ventricular β-receptor density were similar to that reported for chronically hypoxic newborn lambs (2). Our results indicate that the attenuated inotropic response to isoproterenol was not due to down-regulation of left and right ventricular β-adrenergic receptors. It is possible that down-regulation did not occur because catecholamine levels were chronically elevated in hypoxic fetuses (13). Alternatively, in chronically hypoxic newborn lambs, Bernstein et al. 1990 (2) showed that β-receptor density decreased in the left ventricle (exposed to hypoxia alone) but did not change in the right ventricle (exposed to hypoxia and pressure overload). They suggested that the up-regulating effects of pressure overload hypertrophy on the right ventricle compensated for the down-regulating effects of hypoxia and chronically elevated catecholamine levels. In the fetus, both left and right ventricles were exposed to the combined effects of hypoxia and increased arterial pressure. Because the fetal right ventricle is more sensitive to afterload (16, 17, 24, 25), the elevated arterial pressure may have had more of an up-regulating effect on β-receptor density in the right ventricle than in the left ventricle.

Cyclic AMP levels

The fourth major finding in our study was that both isoproterenol-stimulated and forskolin-stimulated cAMP levels were significantly higher in hypoxic fetuses, but basal unstimulated cAMP levels did not differ from normoxic controls. However, the inotropic responses to isoproterenol and forskolin were significantly higher in normoxic fetuses. Together, these results have several important implications. First, these data show that the attenuated inotropic responses to isoproterenol and forskolin were not the result of decreased responsiveness at the level of adenylate cyclase. Secondly, the coupling between surface β-receptors and adenylate cyclase was not altered by long-term hypoxia. These

results strongly suggest that hypoxia acts downstream of second-messenger production in the signal transduction cascade coupled to β-adrenergic receptors. Thus, A-kinase, sarcolemmal L-type Ca^{2+} channels, the ryanodine receptor, phospholamban, troponin-I, and C-protein are all possible sites for the effects of hypoxia. In addition, hypoxia could, theoretically, act at the level of the myofilaments changing the amount of contractile protein, its calcium affinity, ATPase activity, and the rate of cross-bridge cycling.

Previously, we showed that in hypoxic fetuses the inotropic response to extracellular calcium was markedly attenuated, but the density of sarcolemmal calcium channels did not change, and the density of ryanodine receptors increased (6). In addition, the hypoxic ventricles were more responsive to ryanodine. Thus, we speculated that hypoxia may decrease the calcium current and/or the amount of calcium stored in and released by the sarcoplasmic reticulum, thereby attenuating the inotropic response to both calcium and isoproterenol. A reduction in the calcium current might also help to explain the increased production of cAMP in hypoxic fetuses. Yu et al., 1993 (34) showed that the increased influx of calcium via L-type calcium channels in response to isoproterenol or forskolin negatively regulated cAMP levels by feedback inhibition on adenylate cyclase. When extracellular calcium concentration was lowered, or after treatment with calcium channel blockers, the cAMP elevating effect of isoproterenol and forskolin was increased. Further studies are needed to explore the calcium current, and its possible effects on adenylate cyclase activity in fetal sheep exposed to long-term high-altitude hypoxia.

Forskolin-stimulated cAMP levels were significantly higher than isoproterenol-stimulated cAMP levels in both normoxic and hypoxic fetuses. However, the inotropic response to isoproterenol was greater, particularly in hypoxic fetuses. These

results are consistent with the observations of England and Shahid, 1987 (10) discussed above, and suggest that cAMP may be compartmentalized in the ovine fetal heart. Furthermore, our data suggest that although long-term hypoxia increased adenylate cyclase activity, proportionately less of the forskolin-stimulated cAMP was available to A-kinases coupled to the contractile response in hypoxic fetuses than in normoxic fetuses. This suggests that there are other cAMP dependent pathways whose activity may have been up-regulated by exposure to long-term hypoxia.

Differential regulation of left and right ventricles

In normoxic fetuses, there was differential sensitivity to β-stimulation in the left and right ventricles. The right ventricle was significantly more sensitive to isoproterenol, as indicated by the lower EC_{50} values for all three measures of contractility (Table 7), even though β-receptor density was ~25% lower than in the left ventricle (Fig. 9 and Table 9). At the same time, the right ventricle was less sensitive to forskolin, as indicated by the higher EC_{50} values for all three measures of contractility (Table 8). Because isoproterenol-stimulated and forskolin-stimulated cAMP levels, and the maximum inotropic response to forskolin (Fig. 8) were not different between the left and right ventricles, it is likely that the difference in sensitivity exists at the level of A-kinase and/or the effector proteins.

At a maximally stimulating dose of isoproterenol, $-dT/dt_{max}$ was ~2.4 times higher in the left ventricle, but T_{max} and $+dT/dt_{max}$ were not significantly different between the two ventricles (Fig. 6 and Table 6). It is not clear why the maximum rate of relaxation ($-dT/dt_{max}$) was higher in the left ventricle. Physiologically, the rate of relaxation is related to the phosphorylation states of troponin-I and C-protein, and phospholamban.

It is possible, therefore, that A-kinase activity may be differentially regulated in the left and right ventricles. Faster rates of relaxation decrease wall tension thereby reducing metabolic demand. The higher rate of relaxation in the left ventricle may, therefore, represent a physiological adaptation by which the term fetal heart is readied for the transition from intrauterine to extrauterine life.

In hypoxic fetuses, there were no differences in the inotropic responsiveness or sensitivity to isoproterenol between the left and right ventricles. However, the total number of β-receptors was higher in the left ventricular free wall (~85 pmol vs. 73 pmol), even though the density (Fig. 9 and Table 9) was somewhat higher in the right ventricle. In addition, both isoproterenol-stimulated and forskolin-stimulated cAMP levels were significantly higher ($P<0.01$) in the hypoxic left ventricle (Fig. 10), indicating differential regulation of adenylate cyclase.

Conclusion

There is marked attenuation of the positive inotropic responses to isoproterenol and forskolin, and a significant increase in sensitivity to isoproterenol in fetal sheep exposed to high-altitude during days 30–142 of gestation. The decrease in inotropic responsiveness to β-stimulation is not due to down-regulation of myocardial β-adrenergic receptors, or to decreased adenylate cyclase activity. Our results strongly suggest that hypoxia acts down-stream of the second messenger, cAMP, possibly by decreasing A-kinase activity and/or the functional states of key effector proteins, including the sarcolemmal L-type calcium channel, the ryanodine receptor, troponin-I, C-protein, and phospholamban. The changes in inotropy and cAMP levels are consistent with our previous report (6)

which suggested that calcium influx and/or storage and release from the sarcoplasmic reticulum may be decreased. Alternatively, phosphodiesterase activity and phosphatase activity may be altered by long-term hypoxia. These changes may represent cardioprotective adaptations which limit metabolic demand by reducing contractility. Further studies are needed to examine phosphodiesterase, A-kinase and phosphatase activities, the function of key effector proteins, and to directly assess the calcium current and intracellular calcium transient.

Acknowledgments

The authors gratefully acknowledge Al VanVarick, David Trydahl, and the White Mt. Research Station staff for their expert technical assistance. This work was supported by USPHS grants: HD22190 and HD03807.

References

1. Alexander, J. K., L. H. Hartley, M. Modelski, and R. F. Grover. Reduction of stroke volume during exercise in man following ascent to 3,100 m altitude. *J. Appl. Physiol.* 23: 849–858, 1967.

2. Bernstein, D., E. Voss, S. Huang, R. Doshi, and C. Crane. Differential regulation of right and left ventricular β-adrenergic receptors in newborn lambs with experimental cyanotic heart disease. *J. Clin. Invest.* 85: 68–74, 1990.

3. Bristow, M. R., R. Ginsburg, A. Strosberg, W. Montgomery, and W. Minobe. Pharmacology and inotropic potential of forskolin in the human heart. *J. Clin. Invest.* 74: 212–223, 1984.

4. Bristow, M. R., R. Ginsburg, W. A. Winobe, R. S. Cubicciotti, W. S. Sageman, K. Lurie, M. E. Billingham, D. C. Harrison, and E. B. Stinson. Decreased catecholamine sensitivity and β-adrenergic receptor density in failing human hearts. *N. Engl. J. Med.* 307: 205–211, 1982.

5. Brook, W. H., S. Connell, J. Cannata, J. E. Maloney, and A. M. Walker. Ultrastructure of the myocardium during development from early fetal life to adult life in sheep. *J. Anat.* 137(4): 729–741, 1983.

6. Browne, V. A., V. M. Stiffel, W. J. Pearce, L. D. Longo, and R. D. Gilbert. Activator calcium and myocardial contractility in fetal sheep exposed to long-term high-altitude hypoxia. *Am. J. Physiol.* (in press).

7. Cheng, J. B., A. Goldfien, L. E. Cornett, and J. M. Roberts. Identification of β-adrenergic receptors using [3H] dihydroalprenolol in fetal sheep heart: direct evidence of qualitative similarity to the receptors in adult sheep heart. *Pediatr. Res.* 15: 1083–1087, 1981.

8. Dale, P. S., C. A. Ducsay, R. D. Gilbert, B. J. Koos, L. D. Longo, and G. G. Power. A microcomputer program for real time data acquisition in the perinatal physiology laboratory. *J. Dev. Physiol.* 11: 56–61, 1989.

9. Doshi, R., E. Strandness, and D. Bernstein. Regulation of atrial autonomic receptors in experimental cyanotic heart disease. *Am. J. Physiol.* 261 (*Heart Circ. Physiol.* 30): H1135–H1140, 1991.

10. England, P., and M. Shahid. Effects of forskolin on contractile responses and protein phosphorylation in the isolated perfused rat heart. *Biochem. J.* 246: 687–695, 1987.

11. Feldman, A. M. Modulation of adrenergic receptors and G-transduction proteins in failing human ventricular myocardium. *Circulation* 87[Suppl IV]: IV-27–IV-34, 1993.

12. Hartley, L. H., J. A. Vogel, and J. C. Cruz. Reduction of maximal exercise heart rate at altitude and its reversal with atropine. *J. Appl. Physiol.* 36: 362–365, 1974.

13. Harvey, L., R. D. Gilbert, L. D. Longo, and C. Duscay. Changes in ovine fetal adrenocortical responsiveness after long-term hypoxemia. *Am. J. Physiol.* 264: E741–747, 1993.

14. Hultgren, H. N., and R. F. Grover. Circulatory adaptation to high altitude. *Annu. Rev. Med.* 19: 119–127, 1968.

15. Kacimi, R., J. P. Richalet, A. Corsin, I. Abousahl, and B Crozatier. Hypoxia-induced down regulation of beta-adrenergic receptors in rat heart. *J. Appl. Physiol.* 73(4): 1377–1382, 1992.

16. Kamitomo, M., J. G. Alonso, T. Okai, L. D. Longo, and R. D. Gilbert. Effects of long-term, high-altitude hypoxemia on ovine fetal cardiac output and blood flow distribution. *Am. J. Obstet. Gynecol.* 169:701–707, 1993.

17. Kamitomo, M., L. D. Longo, and R. D. Gilbert. Right and left ventricular function in fetal sheep exposed to long-term high-altitude hypoxemia. *Am. J. Physiol.* 262: H399–H405, 1992.

18. Kamitomo, M., T. Ohtsuka, and R. D. Gilbert. Effects of isoproterenol on the cardiovascular system of fetal sheep exposed to long-term high-altitude hypoxemia. *J. Appl. Physiol.* 78(5): 1793–1799, 1995.

19. Lowry, O.H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265–275, 1951.

20. Maher, J. T., J. C. Denniston, D. L. Wolfe, and A. Cymerman. Mechanism of the attenuated cardiac response to β-adrenergic stimulation in chronic hypoxia. *J. Appl. Physiol.: Respirat. Environ. Exercise Physiol.* 44: 647–651, 1978.

21. Maher, J. T., S. C. Manchanda, A. Cymerman, D. L. Wolfe, and L. H. Hartley. Cardiovascular responsiveness to β-adrenergic stimulation and blockade in chronic hypoxia. *Am. J. Physiol.* 228: 477–481, 1975.

22. Oparil, S., S. P. Bishop, and F. J. Clubb. Myocardial cell hypertrophy or hyperplasia. *Hypertension* [Suppl III]: III-38–III43, 1984.

23. Padbury, J. F., A. H. Klein, D. H. Polk, R. W. Lam, C. Hobel, and D. A. Fischer. Effect of thyroid status on lung and heart beta-adrenergic receptors in fetal and newborn sheep. *Dev. Pharmacol. Ther.* 9: 44–53, 1986.

24. Pinson, C. W., M. J. Morton, and K. L. Thornburg. An anatomic basis for fetal right ventricular dominance and arterial pressure sensitivity. *J. Dev. Physiol.* 9: 253–269, 1987.

25. Pinson, C. W., M. J. Morton, and K. L. Thornburg. Mild pressure loading alters right ventricular function in fetal sheep. *Circ. Res.* 68: 947–957, 1991.

26. Richalet, J. P., P. Larmignat, C. Rathat, A. Keromes, P. Baud, and F. Lhoste. Decreased cardiac response to isoproterenol infusion in acute and chronic hypoxia. *J. Appl. Physiol.* 65:1957–1961, 1988.

27. Seamon, K., and J. W. Daly. Forskolin, cyclic AMP and cellular physiology. *TIPS* ?: 120–123, March 1983.

28. Schumacher, C., H. Becker, R. Conrads, U. Schotten, S. Pott, M. Kellinghaus, M. Sigmund, F. Schondube, C. Preusse, and H. D. Schulte. Hypertrophic cardiomyopathy: a desensitized cardiac beta-adrenergic system in the presence of normal plasma catecholamine concentrations. *Naunyn-Schmieddebergs-Arch-Pharmacol.* 351(4): 398–407, 1995.

29. Smolich, J. J., A. M. Walker, G. R. Campbell, and T. M. Adamson. Left and right ventricular myocardial morphometry in fetal, neonatal, and adult sheep. *Am. J. Physiol.* 257: H1–H9, 1989.

30. Smolich, J. Morphology of the developing myocardium. In: *Research in Perinatal Medicine(V). Perinatal Development of the Heart and Lung*, edited by J. Lipshitz, J. Maloney, C. Nimrod, and G. Carson. Ithaca, N.Y.: Perinatology Press, 1987, 1–22.

31. Teitel, D. F., D. Sidi, D. Bernstein, M. A. Heymann, and A. M. Rudolph. Chronic hypoxemia in the newborn lamb: cardiovascular, hematopoietic, and growth adaptations. *Pediatr. Res.* 19: 1004–1010, 1985.

32. Voelkel, N. F., L. Hegstrand, J. T. Reeves, I. F. McMurty, and P. B. Molinoff. Effects of hypoxia on density of β-adrenergic receptors. *J. Appl. Physiol.: Respirat. Environ. Exercise Physiol.* 50(2): 363–366, 1981.

33. Webster, K. A. and N. H. Bishopric. Molecular regulation of cardiac myocyte adaptations to chronic hypoxia. *J. Mol. Cell. Cardiol.* 24 (7): 741–752, 1992.

34. Yu, H. J., H. Ma, and R. D. Green. Calcium entry via L-type calcium channels acts as a negative regulator of adenylyl cyclase activity and cyclic GMP levels in cardiac myocytes. *Mol. Pharmacol.* 44(4): 689–693, 1993.

CHAPTER FOUR

SUMMARY OF MAJOR FINDINGS

This project is part of an ongoing series of investigations focused on the mechanisms by which the mammalian fetus adapts to long-term intrauterine hypoxia. It builds on the work of many investigators, who, in the last 25 years, have described the mechanisms by which cardiac performance is regulated in fetal sheep. Recent studies in our laboratory explored the roles of preload, afterload, and heart rate *in utero* in the regulation of cardiac output in chronically hypoxic fetal sheep. This project examines the role of contractility, as a determinant of cardiac performance, and provides insight into the mechanisms by which fetal myocardial contractility is regulated during long-term high-altitude hypoxia. Specifically, it provides insight into two pathways that are central to the control and regulation of excitation-contraction coupling: calcium sensitivity and handling, and β-adrenergic receptor function.

The major findings are summarized below:

1. Long-term high-altitude hypoxia decreased the inotropic responsiveness to extracellular calcium, but did not change the *number* of sarcolemmal calcium channels, suggesting that hypoxia may, instead, alter the *functional state* of the calcium channel, thereby, decreasing the calcium current, I_{Ca} , and reducing the delivery of calcium to the myofilaments. Because calcium handling requires a significant fraction of the cell's energy budget (~25%), reduced calcium availability may represent an adaptation which lowers metabolic demand in the face of chronically reduced arterial oxygen tension.

2. The force-extracellular calcium relationship was shifted to the left, suggesting that sensitivity to calcium was increased at one or more critical steps in the excitation-contraction coupling cascade. If calcium delivery to the myofilaments is decreased, as speculated, increased sensitivity to calcium may compensate for reduced calcium availability, particularly at physiological serum calcium concentrations.

3. The sarcoplasmic reticulum was the main source of activator calcium in late-term fetal sheep. Exposure to chronic hypoxia increased the functional responsiveness to ryanodine, indicating that the sarcoplasmic reticulum remained the chief source of activator calcium. In addition, the number of ryanodine receptors, and the ryanodine receptor:dihydropyridine receptor ratio increased significantly in both ventricles, indicating that hypoxia did not delay the maturation of two components critical for calcium-induced calcium release: sarcolemmal calcium channels and ryanodine receptors. Hypoxia, may, however, affect the functional state of these channels.

4. In both ventricles, sensitivity to isoproterenol increased significantly, but there was marked attenuation of the positive inotropic responses to isoproterenol and forskolin.

5. The decreased inotropic responsiveness to β-stimulation was not due to down-regulation of myocardial β-adrenergic receptors, or to decreased cAMP levels, suggesting that adenylate cyclase activity was not decreased. However, a marked reduction in phosphodiesterase activity could produce the same pattern of changes in cAMP levels.

6. In normoxic fetuses, β-adrenergic receptor density was significantly higher in the left ventricle than in the right ventricle, indicating differential regulation of β-receptors.

7. Our results strongly suggest that hypoxia acts down-stream of cAMP in the signal transduction cascade, possibly by decreasing A-kinase activity, or increasing phosphatase activity.

Speculation

The changes observed in intracellular cAMP levels, and the inotropic responses to calcium, ryanodine, isoproterenol, and forskolin may be explained by a scenario in which long-term hypoxia alters A-kinase, phosphodiesterase, and/or phosphatase activities, thus reducing the phosphorylation states of several key proteins. These proposed changes may affect the functional states of the sarcolemmal L-type calcium channel, the ryanodine receptor, troponin-I, C-protein, and phospholamban. Theoretically, I_{Ca} and calcium release from the sarcoplasmic reticulum would be reduced, thereby decreasing the delivery of calcium to the myofilaments, and, therefore, reducing the amplitude of contractions. At the same time, myofilament sensitivity for calcium would, theoretically, increase, a consequence of decreased phosphorylation of troponin-I, thereby shifting the force-Ca^{2+} relationship to the left, and slowing the rate of dissociation of calcium from troponin-C. This, together with the reduced phosphorylation state of phospholamban, would slow the rate of relaxation. In addition, the reduced phosphorylation state of phospholamban and the reduction in calcium influx (I_{Ca}) may result in decreased SR calcium loading, thereby decreasing the amount of calcium available for release from the sarcoplasmic

reticulum. Adenylate cyclase activity, which is negatively modulated by the calcium current, would, in theory, increase, resulting in higher intracellular cAMP levels. These changes may represent cardioprotective adaptations which limit metabolic demand by reducing contractility. Further studies are needed to test these hypotheses.