Brief Definitive Report

CARDIOVASCULAR CHANGES IN CALCIUM-DEFICIENT CHICK EMBRYOS

By ROCKY S. TUAN AND HUNG Q. NGUYEN

From the Department of Biology, University of Pennsylvania, Philadelphia, Pennsylvania 19104

To examine how calcium deficiency may affect embryonic development, we have been studying chick embryos that have been rendered calcium deficient by long-term culture without their eggshells (1–4). The systemic calcium deficiency of these shell-less (SL) embryos is a direct result of the removal of their primary source of calcium, the calcareous eggshell, which is normally mobilized into the embryonic circulation by the placenta-like chorioallantoic membrane during development (4–6). The SL chick embryo thus represents a unique experimental system for studying the relationship between calcium homeostasis and other metabolic and physiological functions. The SL embryo is exclusively deficient in calcium since only the calcareous eggshell is missing and all other nutrient sources are essentially intact (7). Furthermore, the fact that the severe calcium deficiency occurs during rapid embryonic growth and organogenesis obviates its effect and permits the investigator to directly analyze the relationship between calcium homeostasis and physiological functions.

In this study we investigate whether embryonic cardiovascular functions are influenced by calcium homeostais. Using chick embryos as the experimental system, we have compared these functions in SL embryos with those in normal (N) embryos, as well as in SL embryos that have been partially supplemented with calcium (Ca-SL). We present here data that strongly indicate a correlation between calcium deficiency and the pathogenesis of hypertension during embryonic development.

Materials and Methods

Chick Embryos and SL Culture. Fertile White Leghorn eggs obtained from Truslow Farms (Chestertown, MD) were used throughout the study. They were incubated at 37.5°C in a humidified commercial egg incubator. The procedure of SL culture has been described previously (1–4, 8, 9). Briefly, embryonated eggs were cracked open aseptically after 3 d of incubation *in ovo*, and transferred to a hemispherical pouch made of transparent plastic kitchen wrap suspended within a ringstand. The culture was loosely covered with a 100-mm Petri dish lid and then placed in a humidified tissue culture incubator at 37.5°C with constant air flow. In some SL cultures, calcium was supplemented in the form of several drops of a slurry of $CaCO_3$ applied on top of the chorioallantoic

This work was supported in part by National Institutes of Health grants HD-15306, HD-15822, and HD-17887; and by the March of Dimes Birth Defects Foundation (Basic Research grant 1-939). Address correspondence to Dr. Rocky S. Tuan, Dept. of Biology, G5, University of Pennsylvania, Philadelphia, PA 19104-4288.

 J. EXP. MED. © The Rockefeller University Press · 0022-1007/87/05/1418/06 $1.00
Volume 165 May 1987 1418–1423

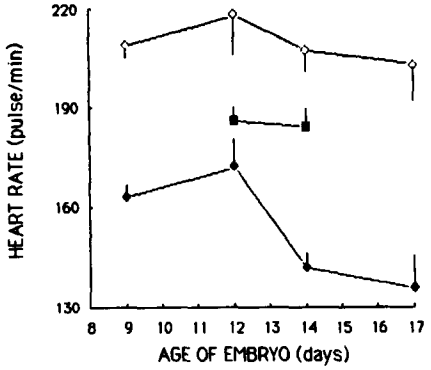

FIGURE 1. Chick embryonic heart rate as a function of development. (◆) N embryo, (◇) SL embryo, (■) Ca-SL embryo. Each value represents the mean ± SD of measurements from 8–12 embryos.

membrane in a manner similar to that previously reported for eggshell supplementation (2, 6, 10).

Heart Rate. Embryonic heart rates were measured at 37°C as the pulse rate of the main chorioallantoic artery. For N embryos, the equatorial region of the eggshell was cracked and partially removed. The chorioallantoic membrane was then lowered using standard egg-windowing procedure (11) to expose the main chorioallantoic artery. For SL embryos, the artery was readily observable because the eggshell was absent. The embryos were maintained at 37 ± 1°C by being placed in a thermostated cup and the arterial pulse rate was measured by observation under a stereoscope.

Arterial Blood Pressure. Blood pressure was measured using a Micropressure System (Model 900; WP Instruments, New Haven, CT). A KCl-filled glass microelectrode with a tip diameter of ~20–50 μm was used to cannulate the main chorioallantoic artery while the embryo was maintained at 36–38°C. Hydrostatic pressures were measured by maintaining the salt concentration gradient at the tip of the microelectrode in dynamic equilibrium by means of a hydraulic air-filled pressurizing system that applied hydrostatic pressures within the electrode equal to the pressures outside. The analog signals (10 mV/mm Hg) were digitally recorded and analyzed using a chromatography software (Chromatochart; Interactive Microware, State College, PA) run on an Apple IIe microcomputer.

Calcium Analysis. Determination of total calcium was carried out using an automated calcium analyzer (CALCETTE; Precision Systems, Inc., Natick, MA) using the fluorescent dye, calcein (3, 4). Samples for calcium analysis included sera (collected from chorioallantoic arteries) and heart tissues after dry-ashing (2, 4).

Results

The first observable sign suggesting cardiovascular pathology in the SL chick embryos is their elevated heart (pulse) rates. As shown in Fig. 1, the heart rates in SL embryos are significantly elevated when compared with the N embryos. During development, the heart rate of the chick embryo normally decreases gradually in an age-specific manner from ~170/min at midgestation to <140/min at late stages of development (day 17). On the other hand, SL chick embryos exhibit obvious tachycardia and the heart rate remains at ~200/min throughout the developmental period observed.

Using a Micropressure System (WP Instruments), we have directly measured the arterial blood pressure in these chick embryos. The data in Fig. 2 clearly demonstrate that the SL embryos are significantly hypertensive. The difference in mean blood pressure between the N and SL embryos results from changes in both the diastolic and systolic pressures, namely, both of these values are higher

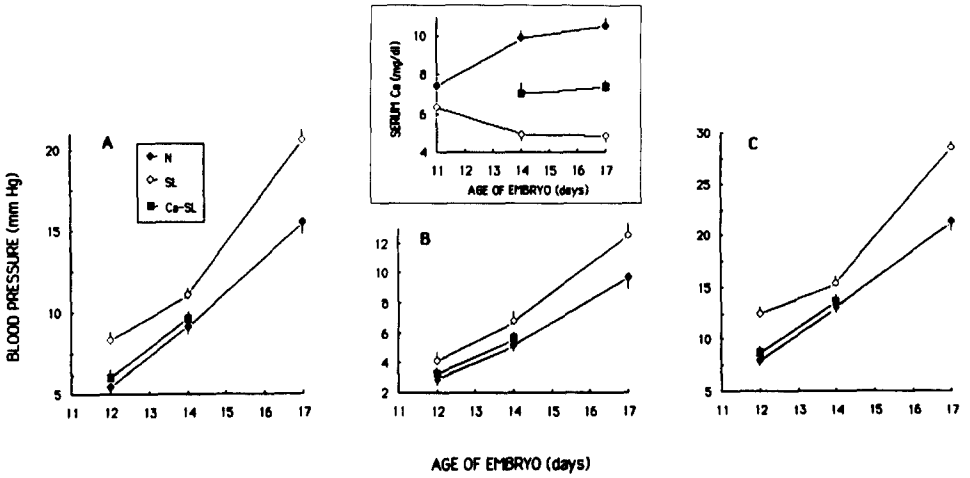

FIGURE 2. Chick embryonic arterial blood pressure as a function of development. (*A*) Mean blood pressure and (*B*) diastolic and (*C*) systolic pressures. (◆) N embryo, (◇) SL embryo, (■) Ca-SL embryo. All values are the mean ± SEM based on >300 pulse height analyses obtained from an average of seven embryos per group. Note that the values on normal embryos reported here are comparable to those previously reported by Van Mierop and Bertuch (29) who used a different instrumental set-up. (*Inset*) Chick embryonic serum calcium level as a function of development. Values are mean ±SEM from >20 embryos at each developmental stage. Data presented are partially based on those previously reported by Ono and Tuan (3).

in the SL embryos. Furthermore, the pulse pressures (i.e., systolic minus diastolic) are also higher in the SL embryos.

A major physiological abnormality of the SL chick embryo is its severe calcium deficiency. For example, as shown in Fig. 2 (inset), the serum calcium level of a day-17 SL embryo, ~5 mg/dl, is only 50% of the normal value. It is thus highly probable that the systemic calcium deficiency and the hypertensive state are causally related. To gain further insight into how the systemic calcium deficiency may have affected cardiovascular and embryonic development in general, we have measured several parameters of embryonic growth and heart development, including total wet weights of embryo and heart, and calcium contents of the embryonic heart. From these data, it is apparent that the overall development of the SL embryo is retarded (Table I), particularly after incubation day 14. Furthermore, in the SL embryo, the embryonic heart is clerly underdeveloped both in terms of its net weight as well as a percentage of total embryo weight (Table I). However, calcium deficiency was not apparent in the heart tissue of the SL embryos. Thus, it is unlikely that the hypertensive state of the SL embryo is a simple consequence of cardiac hypertrophy or abnormal calcium sequestration.

To directly ascertain whether the hypertensive state is functionally linked to the systemic calcium deficiency, we have supplemented the SL embryos with calcium (referred to as Ca-SL embryos), in the form of a $CaCO_3$ slurry applied on top of the chorioallantoic membrane, and have subsequently determined their blood pressure. The results showed that: (*a*) the Ca-SL embryos are able to mobilize the supplemented calcium and their serum calcium approaches a level intermediate between those of N and SL embryos (see Fig. 2, inset); and (*b*) the

TABLE I

Comparison of N and SL Chick Embryos as a Function of Development: Embryonic Weight, and Heart Weight and Calcium Content

Age	Embryo	Weight of embryo	Size of heart		Heart calcium (μg/100 mg)
			Weight	Percent of embryo	
d		*g*	*mg*		
12	N	2.99 ± 0.10 (7)*	32.4 ± 2.6 (7)	1.12 ± 0.06	11.00 ± 0.39 (5)
	SL	3.36 ± 0.20 (8)	31.5 ± 2.0 (8)	0.95 ± 0.07‡	10.98 ± 0.76 (5)
14	N	6.56 ± 0.19 (8)	83.4 ± 2.6 (8)	1.27 ± 0.04	10.61 ± 0.19 (5)
	SL	6.53 ± 0.19 (14)	60.9 ± 3.4 (14)‡	0.93 ± 0.04‡	11.27 ± 0.24 (6)
17	N	13.24 ± 0.43 (20)	155.3 ± 6.8 (20)	1.17 ± 0.04	14.04 ± 0.36 (6)
	SL	10.09 ± 0.70 (7)‡	102.9 ± 2.8 (7)‡	1.04 ± 0.06‡	15.71 ± 0.52 (9)

* Sample size of each analysis is indicated in parentheses.
‡ Values for SL embryos significantly lower ($p \leq 0.05$) than those for N embryos.

supplemented calcium has a significant hypotensive (Fig. 2) as well as bradycardiac (Fig. 1) effect.

Discussion

The results presented here clearly indicate a relationship between calcium homeostasis and cardiovascular functions in the developing chick embryo. The tachycardia and hypertension observed in the SL embryo appear to be a specific result of the calcium deficiency, since calcium supplementation to these embryos significantly restores these functions towards normality. The obvious, intriguing question is: how does systemic calcium deficiency cause such dramatic changes in the cardiovascular functions? Our studies suggest that this is not a simple consequence of abnormal cardiac development or hypertrophy. Preliminary histological observations have also revealed no gross cardiac malformations in the SL embryos. It is highly probable that the hypertensive effect of systemic calcium deficiency is mediated via the alteration of certain physiological or hemodynamic parameters important in the proper maintenance of arterial pressure. For example, sodium balance may have been perturbed in the SL embryo, or vascular resistance is elevated, etc. For this reason, we are currently analyzing other physiological, biochemical, and endocrinological parameters (see discussions in references 12–14) to better define the mechanism of the calcium deficiency–induced hypertension.

Several recent epidemiological and nutritional surveys and studies (15–20) of human populations have suggested an association between low dietary intake of calcium and high blood pressure. There is also additional evidence suggesting a hypotensive effect of calcium loading in patients with essential or mild-to-moderate hypertension (21, 22) and in experimental animals (23, 24). In view of our findings, the SL chick embryo may thus be a potential candidate as an animal model with which to study the relationship between calcium homeostasis and the pathogenesis of hypertension, and complement those presently being used for hypertension studies, e.g., the spontaneously hypertensive rat (25) and the salt-sensitive Dahl rat (26). The singular calcium deficiency of the SL chick embryo had made it an excellent experimental model for studying the functional importance of calcium in membrane transport (1, 2), tissue morphogenesis (3), and

skeletal development (27, 28). We are, of course, aware that such severe calcium deficiency is unlikely in human populations and that the SL embryo thus represents an extreme case of calcium deficiency–mediated hypertension. However, such a condition may be precisely what is needed to experimentally accentuate and make apparent the relationship.

Summary

We have developed an experimental system involving calcium-deficient chick embryos to examine the relationship between calcium homeostasis and cardiovascular activities. We have found that the calcium-deficient embryos, when compared with control animals, exhibit tachycardia and are significantly hypertensive. The effects are unlikely to be due to gross cardiac malformations or hypertrophy. The hypertensive condition appears to be a specific result of the systemic calcium deficiency since calcium supplementation to these embryos significantly restores the functions to normality.

The authors thank Dr. Tamao Ono and Mr. Neil Bigioni for the calcium analysis.

Received for publication 19 November 1986 and in revised form 26 February 1987.

References

1. Tuan, R. 1980. Calcium transport and related functions in the chorioallantoic membrane of cultured shell-less chick embryos. *Dev. Biol.* 74:196.
2. Tuan, R. 1983. Supplemented eggshell restores calcium transport in chorioallantoic membrane of cultured shell-less chick embryos. *J. Embryol. Exp. Morphol.* 74:119.
3. Ono, T., and R. Tuan. 1986. Effect of experimentally induced calcium deficiency on development, metabolism, and liver morphogenesis of the chick embryo. *J. Embryol. Exp. Morphol.* 92:207.
4. Tuan, R., and T. Ono. 1986. Regulation of extraembryonic calcium mobilization by the developing chick embryo. *J. Embryol. Exp. Morphol.* 97:63.
5. Terepka, A., J. Coleman, H. Armbrecht, and T. Gunther. 1976. Transcellular transport of calcium. *Symp. Soc. Exp. Biol.* 58:107.
6. Tuan, R. 1987. Mechanism and regulation of calcium transport by the chick embryonic chorioallantoic membrane. *J. Exp. Zool.* In press.
7. Dunn, B., and M. Boone. 1977. Growth and mineral content of cultured chick embryos. *Poult. Sci.* 56:662.
8. Auerbach, R., L. Kubai, D. Knighton, and J. Folkman. 1974. A simple procedure for the long-term cultivation of chicken embryos. *Dev. Biol.* 41:391.
9. Dunn, B. 1974. Technique for shell-less culture of the 72-hour avian embryo. *Poult. Sci.* 53:409.
10. Ono, T. 1986. Regulation of embryonic calcium metabolism. *Hokuriku J. Zootech. Sci.* 53:37.
11. Coulombre, A. 1967. Grafting of embryonic rudiments. *In* Methods in Developmental Biology. F. Wilt and N. Wessells, editors. Thomas Y. Crowell Co., New York. 457 pp.
12. Lau, K., and B. Eby. 1985. The role of calcium in genetic hypertension. *Hypertension (Dallas).* 7:657.
13. Resnick, L., J. Nicholson, and J. Laragh. 1985. Calcium metabolism and the renin-aldosterone system in essential hypertension. *J. Cardiovasc. Pharmacol.* 7 (Suppl. 6):S187.

14. McCarron, D. 1985. Is calcium more important than sodium in the pathogenesis of essential hypertension. *Hypertension (Dallas).* 7:607.
15. McCarron, D., C. Morris, and C. Cole. 1982. Dietary calcium in human hypertension. *Science (Wash. DC).* 217:267.
16. Ackley, S., E. Barrett-Connor, and L. Suarez. 1983. Dairy products, calcium, and blood pressure. *Am. J. Clin. Nutr.* 38:457.
17. Garcia-Palmieri, M., R. Costas, M. Cruz-Vidal, P. Sorlie, J. Tillotson, and R. Havlik. 1984. Milk consumption, calcium intake, and decreased hypertension in Puerto Rico. *Hypertension (Dallas).* 6:322.
18. Harlan, W., A. Hull, R. Schmouder, J. Landis, F. Thompson, and F. Larkin. 1984. Blood pressure and nutrition in adults. *Am. J. Epidemiol.* 120:17.
19. McCarron, D. 1983. Calcium and magnesium nutrition in human hypertension. *Ann. Intern. Med.* 98:800.
20. Kesteloot, H., J. Geboers, and R. Van Hoof. 1983. Epidemiological study of the relationship between calcium and blood pressure. *Hypertension (Dallas).* 5(Suppl. II):52.
21. Resnick, L., J. Nicholson, and J. Laragh. 1984. Outpatient therapy of essential hypertension with dietary Ca^{2+} supplementation. *J. Am. Coll. Cardiol.* 3:616.
22. McCarron, D., and C. Morris. 1985. Blood pressure response to oral calcium in persons with mild to moderate hypertension. *Ann. Intern. Med.* 103:825.
23. Ayachi, S. 1979. Increased dietary calcium lowers blood pressure in the spontaneously hypertensive rat. *Metabolism.* 28:1234.
24. McCarron, D., P. Lucas, R. Shneidman, and T. Drueke. 1985. Blood pressure development of the spontaneously hypertensive rat after concurrent manipulations of dietary Ca^{2+} and Na^{+}. *J. Clin. Invest.* 76:1147.
25. Okamoto, K., and K. Aoki. 1963. Development of a strain of spontaneously hypertensive rats. *Jpn. Circ. J.* 27:282.
26. Dahl, L., M. Heine, and L. Tassinari. 1962. Role of genetic factors in susceptibility to experimental hypertension due to chronic excess salt ingestion. *Nature (Lond.).* 194:480.
27. Tuan, R., and M. Lynch. 1983. Effect of experimentally induced calcium deficiency on the developmental expression of collagen types in chick embryonic skeleton. *Dev. Biol.* 100:374.
28. Jacenko, O., and R. Tuan. 1986. Calcium deficiency induces expression of cartilage-like phenotype in chick embryonic calvaria. *Dev. Biol.* 115:215.
29. Van Mierop, L., and C. Bertuch. 1967. Development of arterial blood pressure in the chick embryo. *Am. J. Physiol.* 212:43.