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Molecular signals for initiating protein synthesis in organ hypertrophy

(growth/hyperplasia/heart/kidney)

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ABSTRACT **When chronically provoked to increased physiologic activity, organs increase in mass through augmented protein synthesis. This process of compensatory hypertrophy can involve cell division as well as cell growth. To test for molecules that might regulate organ size, by inducing hypertrophy, we performed a series of experiments using isolated, perfused, canine hearts in which the left ventricle was beating but performed no work. Hypertrophying hearts and kidneys as well as normal control organs were extracted and the extracts were perfused through isolated heart preparations. Before and after perfusion, RNA was extracted from fragments of the isolated hearts and translated in cell-free media containing [^{35}S]methionine. Incorporation of methionine into protein was measured by liquid scintillation spectrometry. When perfused through normal hearts, extracts from hypertrophying heart and kidney were able to increase greatly the translational ability of RNA extracted from the normal hearts; corresponding perfusates from nonhypertrophying hearts and kidneys had no effect. Our results indicate that molecules that initiate hypertrophic organ growth are extractable, are generated by the cells of the organ under stress, and are probably similar in heart and kidney and perhaps in many other organs as well.**

When stimulated to increased function, many organs respond by growing larger. Such growth may involve hypertrophy (increased cell size) or hyperplasia (increased cell division) or both (1). The initial stimulus to growth may be mediated through the nervous (2) or endocrine (3) system or by chemical changes produced by the stressed cells themselves. Determination of organ size is critically important to the structure and function of an organism, and the problem of hypertrophic growth regulation has attracted the attention of investigators for many years.

Several hypotheses can be advanced to account for the regulation of organ size: (*i*) stimulating or inhibiting molecules released by the target organ and acting in feedback loops upon the organ have often been suggested as a basic mechanism for fixing organ size in accord with the physiological needs of the organism (4, 5); (*ii*) growth may occur as a consequence of the production of specific kinds of molecules (6, 7) within the target cells themselves without the involvement of any circulating molecules; (*iii*) shifting patterns of metabolic events may bring functional activity and size into concordance, with changes in the proportions of existing molecular species but without the production of regulatory molecules (8, 9). In an attempt to test these various hypotheses and to discover substances that might be involved in regulating organ size, we performed a series of experiments using the canine heart as a target organ.

The muscle cells of mammalian hearts do not increase in number after birth, but they do increase in size in response to increased work loads–that is, the heart hypertrophies. An early biochemical indication of impending hypertrophy is increased RNA synthesis followed by protein synthesis. We measured the initiation of hypertrophy in two different ways: (*i*) by the incorporation of RNA precursors into RNA and (*ii*) by increased translational activity *in vitro* of RNA extracted from the target hearts. Only the results using this second method are reported here. The results of these experiments demonstrate that the hypertrophying heart and kidney both contain extractable molecules that will induce a normal heart to initiate hypertrophy.

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MATERIALS AND METHODS

Left ventricular hypertrophy was initiated in dogs by constricting the ascending aorta so that a 40- to 80-mm Hg gradient was produced between the left ventricular chamber and the systemic circulation. The initiation of hypertrophy was documented by comparing, in the same heart, the amount and kinds of translational products produced in an *in vitro* system by equal amounts of RNA extracted from the right (nonhypertrophying) and left (hypertrophying) ventricles (Fig. 1). Hypertrophying kidneys were obtained from dogs that had been subjected to unilateral nephrectomy 12 hr previously.

Forty-eight hours after aortic constriction or 24 hr after contralateral nephrectomy, the heart or kidney was removed and cleaned of extraneous tissue (i.e., atria, right ventricle, and aorta from the left ventricle; ureter, vascular pedicle, and perinepheric fat from the kidney). The remaining tissue was weighed and homogenized with twice its weight of distilled water in a Waring blender. The homogenate was then centrifuged at 15,000 × *g* for 15 min; the supernatant was decanted and recentrifuged at 30,000 × *g* for another 15 min. The supernatant was then placed in 50-ml plastic syringes, under ice, in a Harvard constant-rate infusion pump for delivery to an isolated heart perfusion preparation over a 6-hr period so that a total of 1 ml of perfusate was delivered per gram of the isolated heart.

Isolated Organ Perfusion Preparation. A freshly extirpated canine heart was used as the target organ for assaying the ability of tissue extracts to stimulate increased translational activity of RNA. In order to maintain normal values of physiologic and biochemical variables such as blood pressure, blood gases, electrolytes, serum osmolarity, and circulating levels of vasoactive, ionotrophic, and chronotrophic substances, etc., an adjacent anesthetized dog was used to support the isolated heart (Fig. 2).

The support animal was anesthetized with sodium pentobarbital, and ventilation was maintained with a Harvard respirator. To prevent blood coagulation, the animal was treated with heparin, 3 mg/kg body weight. Both femoral arteries were exposed and cannulated. One femoral artery cannula was connected to an aortic root cannula with Tygon tubing. The donor dog was similarly anesthetized and heparinized and its

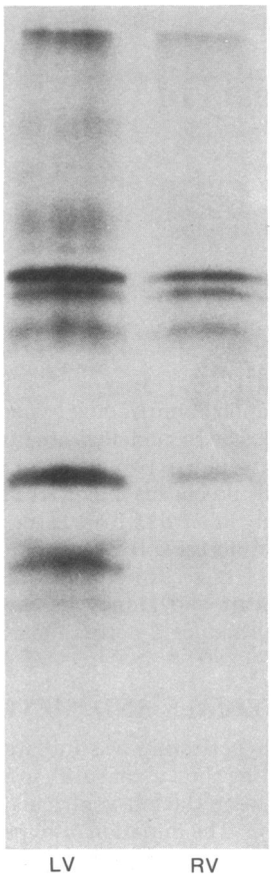

FIG. 1. Translational products produced by normal right (RV) and hypertrophying left (LV) ventricles of a canine heart. Longer exposure of such gels revealed the synthesis of more than 100 distinct proteins.

heart was extirpated. A transmural portion (1–2 g) of free left ventricular wall was removed from the cardiac apex and quick-frozen in liquid nitrogen. The removal of this tissue provided for left ventricular drainage, and the removed tissue served as a control for determinations of RNA translational activity prior to perfusion. The heart was then weighed and connected to the aortic root cannula, and perfusion was started. Because the ventricle had been vented, the heart could generate no pressure and, therefore, performed no work although it was beating. This arrangement obviated the possibility that the experimental heart might begin to hypertrophy in response to an increased work load.

Coronary drainage was collected in a heat exchanger reservoir which maintained temperature at 37°C. The blood was then pumped back into the other femoral artery of the support dog via a roller pump. Arterial pressure for both the isolated heart and the support animal was monitored through a side port in the aortic root cannula and maintained at a mean of 100 mm Hg by varying the rate of infusion of blood back into the support animal. Arterial blood gases were monitored and maintained within normal range by varying the respirator settings. The previously prepared tissue extract was then delivered to the isolated organ through a second port in the aortic root cannula. The preparation was varied in the following ways so that different possibilities for the initiation of hypertrophy could be studied.

Variation 1. To test for the presence of extractable molecules in stressed hearts or kidneys that might initiate hypertrophy in normal organs, soluble extracts were prepared from the left ventricle of a dog that had undergone aortic constriction 48 hr previously or from the remaining kidney after unilateral nephrectomy 24 hr previously. These extracts were perfused through a normal heart.

Variation 2. As a control for the extract of *variation 1*, an extract was prepared in an identical manner except that the heart or kidney that provided the extract had not been induced to hypertrophy.

Variation 3. This variation differed from the two other variations in that no cardiac or renal extract was used. However, the aorta of the support dog was banded 2 days previously. This experiment was performed to determine whether the initiation of hypertrophy in the isolated heart might be due to a generalized circulating substance produced as a result of an operative procedure or to a specific circulating substance released by a heart undergoing hypertrophy as in the support dog.

Variation 4. This variation served as a control for *variation 3* and was identical to it except that the support dog had undergone no previous surgical procedure.

Extraction of RNA for Use in Cell-Free Translation. An important part of our procedure is the isolation of RNA from tissue extracts to measure the translational activity of the RNA in an *in vitro* protein-synthesizing system. The isolation of RNA from heart tissue followed a modification of the procedure used by Ulrich *et al.* (10) which allows for the extraction of intact, translatable RNA from sources rich in RNase. One or 2 g of tissue was diced with scalpel blades, homogenized in 6 M guanidine·HCl/5 mM dithiothreitol/20 mM sodium acetate, pH 5.2, and layered over a cushion of 5.7 M cesium chloride/0.1 M EDTA in a centrifuge tube. After centrifugation for 16 hr at 110,000 × *g*, the RNA pellet was dissolved in water and precipitated overnight with ethanol. The RNA was again collected by centrifugation for 20 min at 17,300 × *g*, dissolved in 2% potassium acetate at pH 5.2, and precipitated overnight by the addition of ethanol (2 vol of absolute ethanol to 1 vol of extract). After collection by centrifugation, the RNA pellet was washed with 80% ethanol, dried, and dissolved in a minimal volume of water for translation. Prior to translation, all RNA samples were diluted to a concentration of 1 mg/ml, on the assumption that 1 A_{260} unit of RNA is equivalent to 50 μg (11).

Rabbit reticulocyte lysate was prepared from washed, packed cells as described by Darnbrough (12) and stored in small portions. The translation mixture contained reticulocyte lysate, 90 mM KCl, 470 μM $MgCl_2$, 9.4 mM creatine phosphate, 40 μg of creatine kinase per ml, 19 unlabeled amino acids (at 26 μM each), 25 μM hemin, and [^{35}S]methionine (≈10 μCi per reaction; 1 Ci = 3.7 × 10^{10} becquerels). The message-dependent translation assays were then performed as described by Pelham and Jackson (13) in a final volume of 46 μl. The amount of [^{35}S]methionine incorporated into protein was determined by trichloroacetic acid precipitation of 5-μl aliquots of translation products on GFA filters and assay in a Beckman LS7000 liquid scintillation spectrometer. Electrophoresis of equal volumes of translation products on acrylamide gels and visualization of the protein bands on the gel by autoradiography provided a visual display of the location and relative amounts of the proteins synthesized *in vitro*.

RESULTS

Translational activity of RNA from the left ventricular myocardium was consistently increased after the heart was perfused with an extract from a hypertrophying left ventricle (Fig. 3). The autoradiogram suggests the degree to which RNA translational activity has been increased and is similar to Fig. 1 which shows the increase in left ventricular RNA translational activity

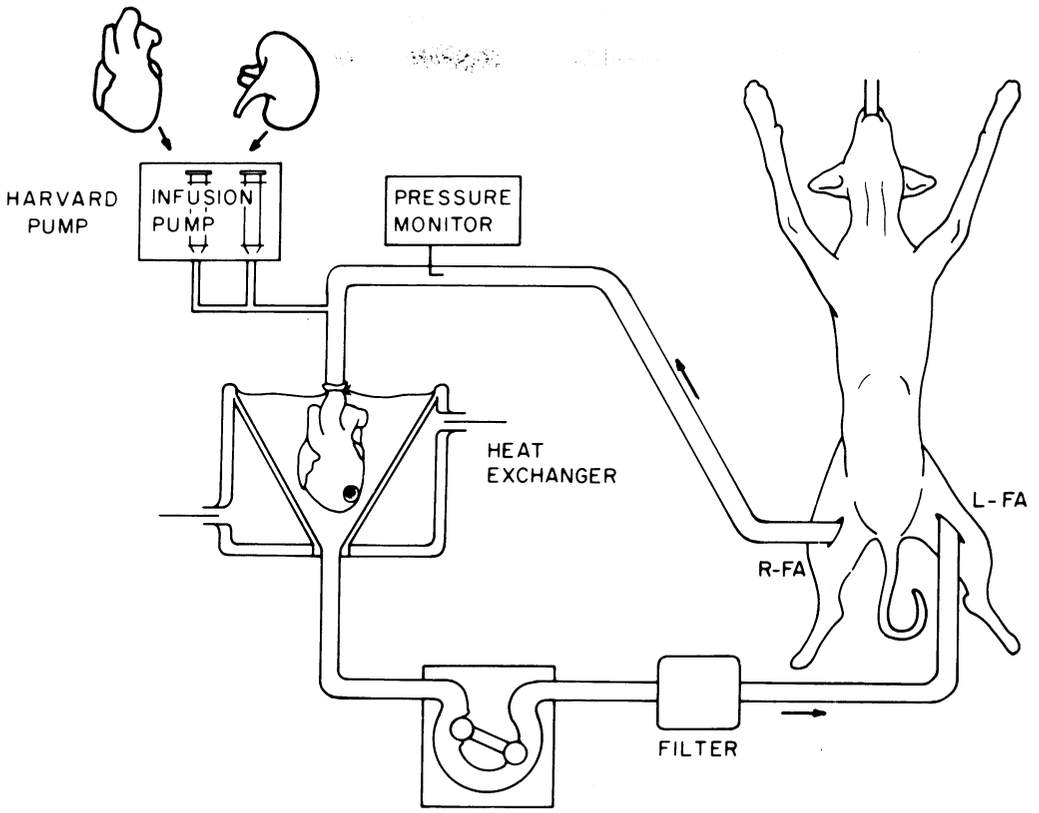

FIG. 2. Diagram of the isolated heart preparation. Extracts from normal and hypertrophying heart and kidney were infused in separate experiments.

of an *in situ* heart that had been subjected to left ventricular outflow tract obstruction.

Fig. 4 quantifies the increase in RNA translational activity in *variation 1* hearts perfused with extract from hypertrophying heart compared with its own preperfusion control and with *variation 2* hearts perfused with nonhypertrophying heart extract and its preperfusion control. Hearts treated by *variations 3* and *4* did not show significant changes in RNA translational activity after perfusion.

When kidney extract was perfused through the isolated heart preparation, extracts from hypertrophying kidney markedly increased cardiac RNA translational activity compared to both its own preperfusion control and the control that had been perfused with extract from a normal kidney (Fig. 5).

The substance(s) that initiated hypertrophy in the *variation 1* preparation was labile when heated to 60°C for 15 min. However, when the extract was stored in a glass container for 24 hr at 4°C, enough activity was retained to provide a satisfactory stimulus for increased translational activity.

DISCUSSION

Although many investigations of cardiac hypertrophy have been performed in the rat, we have found rats not to be satisfactory for these experiments. We were unable to initiate cardiac hypertrophy uniformly and predictably by banding the aorta of rats, and the isolated rat heart perfusion preparations did not function satisfactorily for longer than 1 hr. After 1 hr, we found that the coronary artery flow–pressure relationship changed significantly and that contractility deteriorated. More than 1 hr is usually required to produce a detectable biochemical response that indicates the initiation of hypertrophy (14). We therefore elected to use dogs, which, in our hands, obviated these problems.

During normal embryonic and juvenile development, all organs increase in size in an integrated fashion in accord with the genetic make-up of the individual. Cellular interactions are clearly important in determining relative growth of tissues and organs of the embryo but whether circulating molecules play any significant role is unclear. During juvenile development, circulating hormones play an important role in both absolute growth and relative growth of various tissues and organs. But compensatory hypertrophy or atrophy in the adult seems to require controlling mechanisms that are qualitatively different from those operating in earlier stages of development.

A rather general observation applicable to most organs of the adult is that growth or regression occurs when the functional demand on the organ changes. Accordingly, hypertrophy is seen in the remaining kidney after unilateral nephrectomy and in the heart when a ventricle must pump against increased pressure. An important question is whether the molecular messages that initiate hypertrophy are the same for both organs and, perhaps, for organs in general or whether the molecular signals are specific for each individual organ.

Experiments in paired rats with connected circulations have demonstrated that when the kidneys of one rat are removed the kidneys in the other hypertrophy (15). This had led to the belief that a circulating hormone may be responsible for initiating compensatory growth (15). The same rationale has been used to explain renal hypertrophy in an animal after unilateral nephrectomy (16). Where such a circulating hormone is produced and how it is released are difficult to explain but some investigators exclude the sudden increase in work required of the remaining renal tissue as significant in the hypertrophic response. Their attitude is based on several observations. First, Weinman *et al.* (17) reported that ureteroperitoneal diversion of one kidney produces a marked increase in glomerular filtration and

FIG. 3. Autoradiograms of RNA translation products in equal volumes of lysate. (*A*) From the left ventricle just after the heart had been extirpated. (*B*) From the same left ventricle after the heart had been perfused for 6 hr with an extract from a hypertrophying heart (*variation 1*).

tubular reabsorption of sodium in the intact kidney without a corresponding increase in renal weight or DNA synthesis. Second, Obertop and Malt (18) reported that, when the ureters in one of a pair of connected rats were ligated and the kidneys are left in place, renal tissue in the intact partner will not hypertrophy. However, these investigators (18) and others (19) have also pointed out that the absence of renal hypertrophy after contralateral ureteral ligation is not a universal finding. Nevertheless, these observations have led to the speculation that kidney size is somehow governed by the amount of preexisting renal mass and that the initiation of hypertrophy is perhaps controlled through a negative feedback mechanism involving tissue-specific circulating molecules (4). It must be recognized, however, that measurements on kidneys are obfuscated by an inherent inability to separate the work load from the blood supply.

Despite the arguments against work-induced hypertrophy from renal experiments, it seems to be the only plausible explanation for cardiac or, in fact, any muscular hypertrophy. As illustrated in Fig. 1, when the left ventricle must overcome a gradient by pumping blood through an obstructed aorta, hypertrophy of only this chamber results. Because both ventricles are supplied by blood from the ascending aorta through the coronary arteries, it is difficult to implicate a circulating hormone. Also, because hypertrophy of residual skeletal muscle does not necessarily follow amputation of an extremity, the response, at least in this tissue, does not seem to be related to mass.

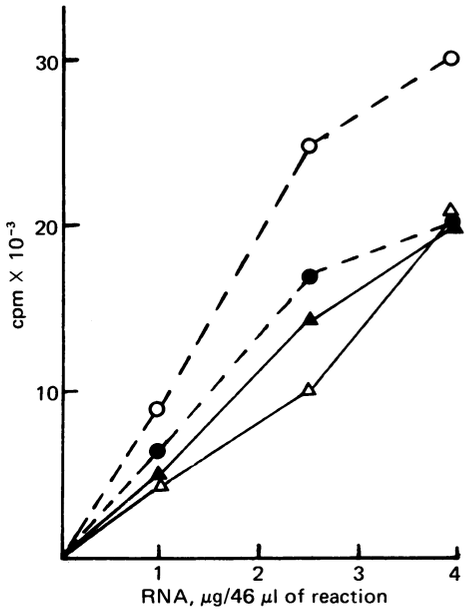

FIG. 4. Increase in RNA activity in *variation 1* hearts perfused with hypertrophying heart extract (O---O) compared with its own preperfusion control (●---●) and with *variation 2* hearts perfused with nonhypertrophying heart extract (△—△) and with its preperfusion control (▲—▲).

A nonspecific circulating hormone that controls cell growth is difficult to postulate, again because both ventricles would hypertrophy when stress was applied to one and because all organs would hypertrophy if a mechanism existed for increasing the circulating levels of such a substance in response to ablation of any organ. On the other hand, if the circulating substance is organ-specific, then serious theoretical problems are raised concerning number of substances, site of production, availability, concentration, etc.

One solution to these problems would be to place the responsible mechanism entirely within individual cells, with no circulating molecules involved at all. This would require the

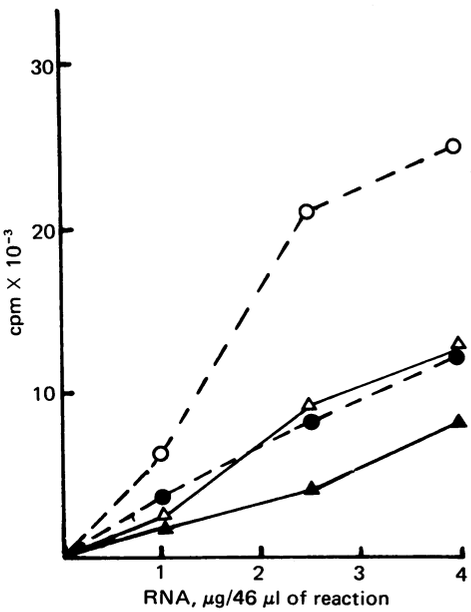

FIG. 5. As in Fig. 4 except that extract was from hypertrophying kidney.

cells of each organ to respond to prolonged increases in the average level of function by initiating DNA synthesis and cell division (hyperplasia) or by increasing protein synthesis (hypertrophy). Our data indicate that the response to stress of both the heart and kidney results in the synthesis of extractable molecules that can initiate the reactions that can lead to hypertrophy. Whether these molecules ever leave the cell in the intact animal is doubtful. Perhaps the molecules produced by the stressed heart that lead to increased cardiac RNA synthesis may also be produced by the stressed kidney. At least extracts from these two organs seem to have the same effect on the heart.

If the stimulus for organ hypertrophy arises and remains within the cell, what change in physiologic activity generates these molecules? Is prolonged physiologic stress biochemically the same in all organs? One common response seems to be an increase in the level of RNA transcription. Our experiments, of course, have only touched on these questions. However, certain speculations can be made from observations of cardiac surgical patients. A common clinical finding in patients with heart disease is that the ventricles of individuals with severe outflow tract obstruction, such as that produced by aortic stenosis, often resemble the ventricles of those patients with triple coronary artery disease. Provided there are no large areas of transmural infarction, the hearts in both clinical conditions may develop fibrotic, noncompliant, hypertrophied ventricles. The observation that ventricular hypertrophy occurs with coronary artery disease, often in the absence of etiological factors such as hypertension, is well documented (20, 21). Cardiac hypertrophy engendered by increased work, as produced in outflow tract obstruction, represents a common adaptive response of muscle tissue, but the occurrence of hypertrophy in coronary artery disease, which produces no obstructive gradient, is more difficult to explain. The common denominator for both pathological conditions, whether produced by decreased blood supply or increased cardiac work, is a prolonged alteration of the normal balance between work load and energy expenditure per cell.

The possibility that this alteration may trigger a series of intracellular biochemical reactions that generate a molecular stimulus for increased RNA transcription and thus protein synthesis and hypertrophy is suggested by previously reported results (22). We noted, as did Bishop and Altschuld (23), that increased right ventricular work produced by pulmonary artery banding produces a shift in the lactate dehydrogenase isozyme pattern toward a distribution rich in A subunits or those that are associated with anaerobic metabolism. We have also shown that the same response of ventricular muscle is seen in patients with coronary artery disease (22). These changes in isozyme pattern must represent prior changes in rates of synthesis or degradation. In the case of the heart, available evidence indicates that hypertrophic growth is a product of increased protein synthesis rather than decreased protein degradation (24). Therefore, it seems likely that changes in isozyme pattern as well as hypertrophy result from alterations in RNA transcription leading to changed or increased protein synthesis.

In this report we have demonstrated the existence of extractable substances specifically produced in hypertrophying organs that can induce the initial reactions of hypertrophy in normal hearts perfused with these extracted substances. This experimental design offers the possibility of identifying and purifying these molecules.

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