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IN VITRO STUDIES OF BEATING HEART CELLS IN CULTURE

XVII THE STIMULATION OF MYOSIN SYNTHESIS

BY A HEAT STABLE SERUM FRACTION¹

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SUMMARY

A heat stable fraction of serum (HSF) maintains the myosin level by stimulation of myosin synthesis. It has little effect on the rate of myosin degradation and appears to act mainly on the transcriptional level.

The myosin level in cultures of neo-natal rat heart cells is maintained by the serum level of the medium (1). The serum acts both by stimulating myosin synthesis and protecting against myosin degradation (2). Fractionation of the serum has led to the separation of a heat stable fraction (HSF) which maintains the level but does not support growth or the growth dependent increase in myosin (3). HSF appears to accomplish this mainly by stimulation of myosin synthesis. It acts on the transcriptional level and has little effect on the degradation.

METHODS

Rat heart cells were cultured and HSF prepared as previously described (4). Ham's F10 (5), (Gibco) was supplemented with antibiotics and 10% fetal calf + 10% horse serum (Microbiological Assn.) and called complete medium. The medium without serum is called minimal medium and minimal medium plus HSF is called HSF medium. A suspension of 5×10^6 cells per 20 x 100 mm plastic petri dish (Falcon) was usually plated in complete medium and in general kept for 2 days before change to experimental media.

For degradation experiments the cells were incubated in complete medium with $1 \mu\text{C}^{14}\text{C}$ leucine per plate (^{14}C leucine (U) 344 m ci/m Mol (Radiochemical Center) long enough to achieve maximal labeling. After the incubation period the medium was aspirated and the cells washed with unlabeled medium containing an excess of unlabeled amino acid. A sample of the cells was taken for a zero point determination and the rest were returned to a base

medium containing 40 mg/liter unlabeled leucine.

The myosin was isolated and determined by acrylamide gel chromatography (2,6). Total protein was collected by TCA precipitation and counted as previously described (6). Radioactivity, expressed as percent of the initial counts, was plotted semilogarithmically against time. From this the half life and β , the decay rate constant, was obtained. ($\beta = 0.693/t^{1/2}$ where $t^{1/2}$ = the half life).

The protein synthesis rate was determined from the absolute amount of protein present at various times and the degradation rate

$$P_s = P_1 - P_o + \beta (P_o)$$

where P_s = μ g of protein synthesized, P_o = initial amount, P_1 = protein level at time t_1 and β = decay rate constant. The synthesis rate was also determined independently by measuring the uptake of labeled amino acid in short pulses at various times in the culture.

RESULTS

The absolute level of myosin was determined by isolation and determination on acrylamide gel. The results from this determination paralleled the results previously reported (3). HSF maintained the myosin level as well as complete medium for 2 days up to the 4th day of culture. In general after this period the HSF medium was not as effective as the complete medium (Table I). Complete medium which contains whole serum protects myosin against degradation and also stimulates synthesis (2). It was, therefore, of interest to measure the effect of HSF on both degradation and synthesis. As compared to minimal medium, HSF had little effect on the degradation of myosin. Complete medium was more effective than HSF (Table II, Exp. 2, 3a, 4) and the effect of HSF was similar to that of minimal medium (Table II, Exp. 2,

3b, 4). On the other hand the rate of myosin synthesis in HSF medium was considerably higher than in minimal medium (Table III, Exp. 1, 2, 4). The rate of myosin synthesis in HSF medium as compared to complete medium was similar (Table III, Exp. 2, 3). This was not true in Exp. 4 where the effect of complete medium on the growth was so great as to possibly introduce other factors in myosin synthesis rate.

The specificity of HSF can be more easily seen when the effect of HSF on myosin synthesis is compared to its effect on total protein. The ratio of synthetic rates in HSF as compared to minimal medium is 2 to 7 fold higher for myosin than for total protein. In addition the whole serum, contained in complete medium, compared to HSF had an equal effect on myosin and a somewhat greater effect on total protein. Thus HSF seemed specific for myosin and total serum had little to add in this regard. The amino acid uptake into myosin was measured directly with pulse experiments. Complete, HSF, and minimal media were compared for their effect (Table IV), HSF stimulated radioactive leucine uptake seven-fold when compared to minimal medium and had an effect equivalent to the complete medium. In comparison, the effect of HSF on total protein synthesis was less than two-fold when compared to minimal medium.

Actinomycin D affected the synthesis of myosin in cells incubated in complete medium but only after 8 hours, indicating a long lived messenger. The effect in HSF was similar where Act. D decreased myosin synthesis sometime just before 8 hours of incubation (Table V). In contrast cycloheximide, directly interfering with protein synthesis, affected myosin synthesis much sooner, since by 8 hours the synthetic ability was completely inhibited (Table VI).

To determine whether the HSF acts directly on the transcriptional level, cells were preincubated with HSF or minimal medium with and without cycloheximide and then washed clean of these media (Table VII). All were then tested in minimal media for their ability to synthesize myosin by use of a ^{14}C leucine uptake into myosin. Two questions were asked in this experiment. One, do cells preincubated with cycloheximide show a decreased ability to synthesize myosin in the post preincubation period? In other words, is protein synthesis necessary for myosin transcription? Two, do cells incubated in HSF acquire and maintain an enhanced ability to synthesize myosin? The assumption in these experiments is that the capacity to synthesize myosin in the minimal medium represents the transcription activity in the preincubation period. When preincubation in HSF was compared to preincubation in HSF plus cycloheximide, the leucine uptake was markedly decreased in the assay period in cells preincubated with cycloheximide. The same result was obtained when preincubation in minimal medium was compared with minimal medium plus cycloheximide. This indicates that protein synthesis is necessary for myosin transcription in the presence or absence of HSF in the preincubation period. Finally if one compares HSF plus cycloheximide to minimal medium plus cycloheximide, one obtains the stimulation of synthesis capacity independent of protein synthesis. In this case HSF stimulated the myosin synthetic capacity almost four-fold. On the other hand, HSF did not appreciably change the uptake into total protein as the count in total protein in cells preincubated in HSF + cycloheximide was 16,290 and for the cells preincubated in minimal + cycloheximide the count was 14,690. In another experiment the counts in myosin in cells preincubated in HSF + cycloheximide were 190 and in minimal medium + cycloheximide 40. At the same time the counts in total protein were 9,340 in the first and 10,420 in the second. Here too, while

preincubation with HSF increased the myosin synthetic capacity over 4-fold, it had no effect on the total protein.

DISCUSSION

The increase in the myosin level caused by HSF (3), appears to be largely a result of a stimulation of myosin synthesis and not an effect of HSF on the degradation rate. In long term experiments, there appears to be an effect of HSF on the counts left in myosin giving such curves a biphasic character. Over a long period of time, the loss in counts in myosin may be masked by changes in the rate of myosin synthesis and the greater reincorporation of isotope in the presence of HSF. The difficulty in measuring degradation rates over long periods of time is well known (7). The same results can also be obtained if one has more than one exponential component (8) or if the myosin molecule decays in a heterogenous fashion (9). Despite these difficulties the degradation rate is not overly affected by the presence of HSF especially during the first days. These data indicate HSF activity is confined to myosin synthesis. Previous experiments also indicate that HSF activity is largely confined to myosin. It has no effect on the levels of creatine phosphokinase, lactic dehydrogenase or on the total protein.

HSF stimulates myosin synthesis 2 to 7 fold more than it stimulates uptake into total protein. This specificity is further supported by the observation that preincubation in HSF plus cycloheximide increased the cellular capacity to synthesize myosin 4-fold as measured within the first 2 hours after the preincubation period. In contrast the same conditions caused no appreciable increase in the capacity to synthesize total proteins. These experiments also indicate that HSF specifically stimulates the transcriptive phase of myosin synthesis. However, it is possible that the lack of effect of HSF on the capacity to synthesize total protein is a result of the short

life of the average heart cell mRNA as compared to the myosin mRNA.

It is also of interest to note that the effect of preincubated HSF on the synthetic capacity, while it can be carried over to the cells replated in minimal media, decreases to 50% of the maximal value within 2 hours. This is faster than one would expect from the Actinomycin D experiment which indicates that the capacity to synthesize myosin is not affected before 8 hours after the addition of the inhibitor.

The relative specificity and its effect on transcription establish the importance of the heat stable factor of the serum in maintaining myosin synthesis. It appears to be nonlipid, nonprotein and of a low molecular weight (3). Further work on the nature of this serum factor is underway.

Table I. Myosin and Total Protein Level

Days 4				6	
(μg/plate)					
	<u>Medium</u>	<u>Myosin</u>	<u>Total Protein</u>	<u>Myosin</u>	<u>Total Protein</u>
Expt 1	HSF	46	400	52	390
	Minimal	"	"	18	320
Expt 2	Complete	60	730	49	900
	HSF	"	"	49	710
	Minimal	"	"	29	490
Expt 3*	Complete	12	620	28	740
	HSF	"	"	24	530
Expt 4	Complete	30	612	90	815
	HSF	"	"	30	400
	Minimal	"	"	15	380

The cells were plated in complete medium, as described in methods, incubated for 2 days and replated for 2 more days with radioactive medium (see Table II). On the 4th day, the myosin level and total protein were determined and the cells transferred to the experimental media designated. * For experiment 3 the figures represent determinations on days 5 and 7. These cells were incubated on radioactive medium for 2 days and shifted on the 5th day.

Table II. Degradation of Total Protein and Myosin

		<u>Medium</u>	Day 6	8
			Percent initial radioactivity	
Expt 1	Total Protein	HSF	29	18
		Minimal	23	10
	Myosin	HSF	24	6
		Minimal	9	0
Expt 2	Total Protein	Complete	41	24
		HSF	35	15
		Minimal	31	10
	Myosin	Complete	44	39
		HSF	32	23
		Minimal	32	23
Expt 3a	Total Protein	Complete	37	37
		HSF	37	36
	Myosin	Complete	86	50
		HSF	40	38
Expt 3b	Total Protein	Complete	28	13
		HSF	33	18
		Minimal	24	18
	Myosin	Complete	27	9
		HSF	14	8
		Minimal	18	--
Expt 4	Total Protein	Complete	62	43
		HSF	42	26
		Minimal	37	13
	Myosin	Complete	76	54
		HSF	31	29
		Minimal	30	12

Experimental conditions as described in Table I. Data expressed as percent of the initial radioactivity (set at 100%) determined on the 4th day except for Expt 3 when the initial count was taken on the 5th day and the next samples on day 7 and 9.

100 per cent counts in total protein is equivalent to 360,510 in Expt 1, 496,117 in Expt 2, 137,062 in Expt 3 and 230,210 in Expt 4. 100% counts in myosin is equivalent to 2,990 in Expt 1, 7,332 in Expt 2, 2,533 in Expt 3, and 8,175 in Expt 4. Expt 3b differs from the others in that the cells after incubation in complete medium for 2 days were treated for 3 days with 10^3 M BUdR and irradiated before switching to the media indicated.

Table III. Synthesis Rates in Culture

		P _s <u>μg/day/plate</u>	
	<u>Medium</u>		
Expt 1	Total Protein	HSF	276
		minimal	269
	Myosin	HSF	26
		minimal	10
Expt 2	Total Protein	Complete	430
		HSF	500
		Minimal	260
	Myosin	Complete	17
		HSF	25
		Minimal	2
Expt 3	Total Protein	Complete	400
		HSF	190
	Myosin	Complete	17
		HSF	20.5
Expt 4	Total Protein	Complete	644
		HSF	167
		Minimal	101
	Myosin	Complete	49
		HSF	20
		Minimal	7

Experimental conditions as described in Table 1. Calculations as described in text. The synthesis rate was calculated for the two day period, 4-6 days of incubation, which represents two days incubation on the experimental media (Except for expt. 3 where the period is 5 to 7 days).

Table IV. Effect of HSF on ^{14}C leucine uptake

	Myosin				Total Protein			
	Level $\mu\text{g}/\text{dish}$		Uptake CPM/dish		Level Mg/dish		Uptake CPM/dish	
	Day 0	2	0	2	0	2	0	2
<u>Medium</u>								
Complete	12	12	2,604	4,737	.887	1.5	21,625	166,925
HSF		14		4,273		0.96		71,620
Minimal		14.5		698		0.76		38,410

Cells were plated in complete medium and incubated for 3 1/2 days. 1 μC of ^{14}C leucine per plate was added to four plates for 2 hours and the uptake into myosin was determined before the media change. This is the control value determined on day 0. The rest of the plates were changed to the experimental media as indicated and incubated for 2 days. ^{14}C leucine was again added for 2 hours and the level and uptake into myosin and total protein was determined as before.

Table V. Effect of Actinomycin D on the HSF Effect

hours	0		4		8		24	
<u>Total Protein</u>								
<u>Medium</u>	<u>CPM</u>	<u>SR</u>	<u>CPM</u>	<u>SR</u>	<u>CPM</u>	<u>SR</u>	<u>CPM</u>	<u>SR</u>
Complete	4,650	22	12,815	52	13,977	56	44,748	132
Complete + Act. D			2,137	13	4,187	18	2,375	11
HSF			5,337	37	5,037	23	7,237	35
HSF + Act. D			4,370	17	2,790	11	731	8
<u>Myosin</u>								
Complete	137	14	241	22	285	15	912	45
Complete + Act. D			169	18	130	9	99	10
HSF			155	17	174	10	225	22
HSF + Act. D			235	16	122	7	66	6

Cells were plated in complete medium and incubated for almost two days. At 46 hours a pulse of ^{14}C leucine (1 μC plate for 2 hours) was given to a sample culture to establish the uptake at zero time in complete medium. At 48 hours the cells were shifted to experimental media as designated, Act. D = 0.2 $\mu\text{g}/\text{ml}$. After 2 hours ^{14}C leucine was added and the myosin and protein was determined at 4 hours. This procedure was repeated at 6-8 and 22-24 hours.

SR=CPM/mg.prot/plate. CPM = CPM/dish.

Table VI. Effect of Cycloheximide on Myosin Synthesis

	hours 0	8	24	48
	Radioactivity (CPM/dish)			
<u>Medium</u>				
Complete	145	107	222	707
Complete + Cycloheximide	145	7	0	5
HSF	145	103	95	85
HSF + Cyclohex- imide	145	0	6	0

As in Table VI, cells were incubated for 2 days on complete medium then shifted to experimental media. Cycloheximide = $2\mu\text{g/ml}$. ^{14}C leucine was added 2 hours before indicated time and the value represents a 2 hour uptake of the amino acid ending with the time indicated.

Table VII. The Effect of Preincubation on Synthesis of Myosin

Preincubation Medium	hour	Myosin Radioactivity CPM/dish		
		12	14	16
Complete*		394	263	244
HSF		382	315	131
Minimal		265	177	30
HSF + Cycloheximide		190	102	40
Minimal + Cycloheximide		40	30	30

The cells were plated and incubated in complete medium for 2 days, washed and transferred to the preincubation medium for 12 hours. In one complete set of samples, the protein synthesis during the preincubation period of 12 hours was measured by the addition of a pulse of ^{14}C leucine from 10 to 12 hours (values in first column). At 12 hours the cells were washed with minimal medium and all dishes except the line labeled "complete" (see below) were reincubated in minimal medium. ^{14}C leucine was added to one set in minimal medium, the cells incubated for 2 hours and assayed, at 14 hours. Another set of cells were incubated for 2 hours in minimal medium and at 14 hours ^{14}C leucine was added and the cells reincubated for a further 2 hours, to 16 hours. Thus, the determination of radioactivity in myosin and total protein in 14 and 16 hours represents the uptake of ^{14}C leucine for 2 and 4 hours in minimal medium in cells which had been preincubated in the experimental media listed. The determination in 12 hours represents the synthetic ability of the cells during their stay in the preincubation media.

*As a control cells preincubated in the complete medium were allowed to remain in complete medium throughout the experiment. Cycloheximide = 2 $\mu\text{g/ml}$. The myosin was measured as already described.

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