THE EFFECT OF GLUCAGON ON DNA SYNTHESIS IN RAT SPLEEN AND BONE MARROW

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1. Introduction

It has been demonstrated that glucagon will enhance the rate of radioactive thymidine incorporation into DNA in foetal and postnatal rat liver *in vitro*. The effectiveness of the hormone is age dependent, and declines postnatally [1]. There is a good correlation between the diminishing glucagon effect and the reduction in erythropoietic content of foetal rat liver [2, 3]. During the development of the rat embryo, the sites of maximal erythropoietic activity are the yolk sac, the liver, the spleen and the bone marrow in chronological order [3]. This communication reports the results of experiments on the effect of glucagon on DNA synthesis in non-hepatic erythropoietic tissue in the rat.

2. Methods

The preparation of spleen slices, conditions for incorporation of ^{3}H-thymidine into DNA, and the extraction of DNA for specific radioactivity determination is as described for foetal liver studies [1]. Spleens from 4 littermate (9 day old) animals were pooled for each determination in order to have suffifient tissue for DNA extraction. In the case of foetal animals where the organ was considerably smaller, the experiments were carried out on diced spleen instead of slices. A glucagon concentration of 75 μg/ml, which was previously shown to yield a maximal effect in liver slices, [1] was used for these experiments.

Bone marrow was obtained from adult male rats by flushing the femur and tibia bones with cold Krebs ringer phosphate buffer pH 7.4 from a syringe. The cells were concentrated by centrifugation at 5° and the total yield from each animal made up to 3 ml. The tissue was thoroughly dispersed by repeated aspirations using a syringe. 1 ml aliquots were taken for incorporation studies and 0.5 ml of the remainder was extracted with 0.5 N $HClO_4$ (final concentration) at 90° for 15 min. The DNA content of aliquots of the extract was determined by the diphenylamine method of Burton [4]. For the time course experiment, the total bone marrow from 3 animals was pooled. Glucagon (50 μg/ml final conc.) or saline was introduced at the commencement of the 5 min preincubation period. The reaction was started by the addition of ^{3}H-thymidine (2.7 nmoles, 1 μCi). Routine incubations were for 10 min at 37° in a shaking water bath (120 oscillations/min). The reaction was terminated by addition of 200 μl of 3 N $HClO_4$. The precipitate was then washed by resuspension and centrifugation (15 sec at 1000 *g*) 5 times with 0.5 N $HClO_4$ (3 ml). This procedure removed all unincorporated counts since blanks which received 3 N $HClO_4$ prior to ^{3}H-thymidine contained no counts above the background level.

The washed pellet was dissolved in 1 M hyamine hydroxide (0.5 ml), mixed with 10 ml of diotol [5] and radioactivity determined in a Nuclear Chicago Model 6860 liquid scintillation spectrometer. Quench corrections were made by the channels ratio method and count rates were adjusted to a standard 21% efficiency. The results are expressed as cpm/mg DNA. The specific activity of DNA isolated from bone marrow cells exposed to ^{3}H-thymidine under similar conditions compared favourably with the specific activities obtained using this simpler procedure.

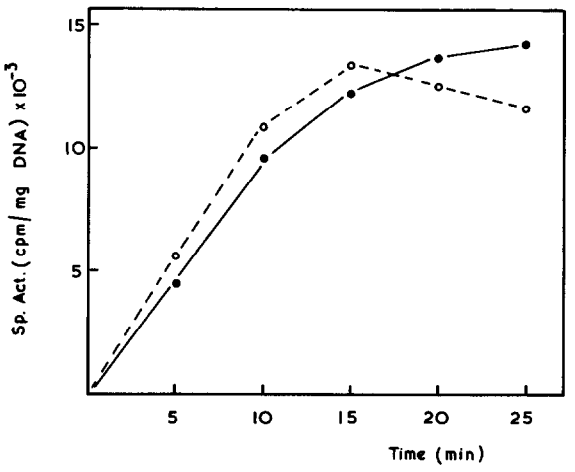

Fig. 1. Effect of glucagon on incorporation of ^{3}H-thymidine into DNA of bone marrow cells. The conditions of assay are as described in the Methods section. (●–●–●): Represents control flasks; (○– –○– –○): represents glucagon (50 μg/ml) treated flasks.

Table 1
The effect of glucagon on ^{3}H-thymidine incorporation into DNA of 9½ day old rat spleen.

	Specific activity of DNA (cpm/mg DNA)	
	Control	Glucagon
	7,622	9,052
	9,104	11,984
	9,820	11,033
	8,635	10,592
	7,622	8,382
	9,094	13,804
Mean	8,650	10,800
± SE	360	810
p value:		$0.02 > p > 0.01$

Glucagon (75 μg/ml) was introduced at the commencement of the preincubation period (10 min). ^{3}H-thymidine (1 μCi/2.7 nmoles) was added to start the reaction. A 20 min incubation was used routinely. Statistical analysis was by the paired difference *t* test.

Table 2
The effect of glucagon on ^{3}H-thymidine incorporation into DNA of foetal rat spleen.

	Specific activity of DNA (cpm/mg DNA)	
	Control	Glucagon
	47,796	50,466
	33,742	36,850
	47,249	50,741
	53,298	50,358
	38,952	38,643
Mean	44,208	45,411
± SE	3,478	3,143
p value:		$0.4 > p > 0.3$

Animals were between 21 and 22 days gestational age. Glucagon (75 μg/ml was introduced at the commencement of the preincubation period (10 min). ^{3}H-thymidine (1 μCi/2.7 nmoles) was added to start the reaction. A 20 min incubation was used routinely. Statistical analysis was by the paired difference *t* test.

Table 3
The effect of glucagon on ^{3}H-thymidine incorporation into DNA of adult rat bone narrow.

	Specific activity of DNA (cpm/mg DNA)	
	Controls	Glucagon
	9,497	12,677
	11,763	12,942
	11,911	12,053
	9,726	10,737
	9,120	9,690
	10,790	11,710
Mean	10,468	11,635
± SE	489	502
p value:		$0.05 > p > 0.02$

Conditions of assay are as described in the methods. Glucagon (50 μg/ml) was introduced at the commencement of the 50 min preincubation period. Statistical analysis was by the paired difference *t* test.

3. Results

Table 1 shows that glucagon stimulates ^{3}H-thymidine incorporation into DNA in spleen preparations from 9 day old rats *in vitro*. Statistical analysis (t test) reveals that controls and tests are significantly different ($0.01 < p < 0.02$). Table 2 summarizes results from foetal spleen where no significant stimulation of DNA synthesis was found. Similar data was obtained when 1 day postnatal spleen was studied.

Fig. 1 shows a typical time course of ^{3}H-thymidine incorporation into DNA of bone marrow cells in the absence and presence of glucagon. Characteristically, when the incubation period is extended beyond the linear region, the specific activity of DNA in glucagon treated flasks is less than that of controls. The effect of glucagon is marginal (11% increase) for incubations within the linear region of the time course. Analysis of the results from 6 experiments gives a p value of between 0.05 and 0.02 (table 3).

4. Discussion

A study of hormonal effects on DNA synthesis in neonatal rat liver slices revealed a stimulatory effect of glucagon which was age dependent [1]. Data suggested that in the course of development, the foetal liver lost its ability to respond to the hormone. It is well established that foetal liver is a major site of haemopoiesis and that its erythropoietic role diminishes with age [2, 3]. The decline in haemopoietic cell content of foetal liver parallels the reduction in effectiveness of glucagon which occurs postnatally, and thus the effect may be confined to the erythropoietic component of neonatal liver.

Data obtained from studies on other erythropoietic tissue, i.e., the spleen and bone marrow, could thus provide evidence in support of the proposed cellular site of glucagon action. These investigations reveal that glucagon significantly elevated DNA synthesis in spleen from $9\frac{1}{2}$ day old rats. At this age, the tissue exhibits maximal erythropoietic activity. Furthermore, foetal spleen, which has a higher rate of DNA synthesis, though considerably less erythropoietic activity, is not affected by glucagon (table 2).

Due to the scarcity of tissue it was not possible to examine the effect of glucagon on bone marrow at the time of maximal erythropoiesis (about 10 days postnatal). Bone marrow erythropoiesis gradually declines in postnatal life in contrast to the liver and spleen [3]. Studies using adult rat bone marrow show that a significant, though marginal, elevation of ^{3}H-thymidine incorporation results from treatment with glucagon.

The level of stimulation observed for each of the haemopoietic tissues varies (liver, 47%; spleen, 25%; bone narrow, 11%). Preliminary autoradiographic data indicates that in foetal liver the hormonal effect may be restricted to a minor cell population. The varying effectiveness of glucagon possibly reflects the relative proportion of target cells in each of the tissues.

Current studies in foetal rat liver have failed to demonstrate an effect of the hormone on enzymes involved in the incorporation of thymidine into DNA [6]. It has been suggested by several investigators that endonuclease [5] may play an important role in regulation of DNA synthesis [7, 8, 9]. The enzyme can determine the state of the primer [10], the availability of 3′-OH ends as well as the rate of degradation of product. The time course of ^{3}H-thymidine incorporation into DNA (fig. 1) repeatedly shows an initial stimulation by glucagon but an inhibitory effect at extended incubation times. This paradoxical effect of the hormone suggests the possibility that it is acting by enhancing the activity of an endonuclease. Initially, this may promote the activity of the polymerizing enzyme by increasing the availability of 3′-OH ends and/or by altering the state of the primer DNA (if the polymerase prefers denatured primer). Subsequently the endonuclease attains a level of activity whereby its degradative role predominants, thus accounting for the lower specific activity of glucagon treated tissue at 20 and 25 min incubation times.

The results of this study lend support to the proposal that the cellular site of glucagon action in foetal rat liver may be the haemopoietic cells.

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References

[1] G. Yeoh and I.T. Oliver, Comp. Biochem. Physiol. 39A (1971) 723.
[2] I.T. Oliver, W.F.C. Blumer and I.T. Witham, Comp. Biochem. Physiol. 10 (1963) 33.
[3] G. Lucarelli, D. Howard and F. Stohlman, J. Clin. Invest. 43 (1964) 2195.
[4] K. Burton, Biochem. J. 62 (1956) 315.
[5] R.J. Herberg, Anal. Chem. 32 (1960) 42.
[6] G. Yeoh and I.T. Oliver, Int. J. Biochem., in press.
[7] E. Baril, O. Brown and J. Laszlo, Biochem. Biophys. Res. Commun. 43 (1971) 754.
[8] L.A. Burgoyne, M.A. Wagar and M.R. Atkinson, Biochem. Biophys. Res. Commun. 39 (1970) 254.
[9] W.E. Lynch, R.F. Brown, S. Langreth, T. Umeda and Lieberman, J. Biol. Chem. 245 (1970) 3911.
[10] M. Hyodo and T. Ono, Biochem. Biophys, Acta 213 (1970) 228.