ACTION OF HEMIN ON CHROMATIN PROTEIN KINASES OF RAT LIVER

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1. Introduction

Hemin inhibits indirectly or directly the activity of a protein kinase (of phosvitin type) independent from cyclic AMP associated with ribosomes in rabbit reticulocytes [1–3] and with microsomes in mouse liver [4].

The non-histone proteins of chromatin contain protein kinases independent from cyclic nucleotides which phosphorylate endogeneous proteins as well as phosvitin and casein [5–7].

The activities of these nuclear kinases vary according to physiological circumstances [8,9] and the cell cycle [10,11] (for reviews see ref. [8–11]). The mechanisms of regulation of these enzymes are unknown. This is why we have found it interesting to examine whether these kinases are also inhibited by hemin and whether this inhibition is reversed by some interferon inducers as it is the case for the mouse liver microsomal protein kinase.

In this work, we show that hemin, at concentrations ranging from 20–100 μM, inhibits 80% of the activity of rat liver tested on non-histone proteins of chromatin as well as phosvitin and casein. The interferon inducers tilorone and quinacrine reverse the inhibition caused by hemin. The possible implications of these findings in the mechanism of nuclear inactivation of the red cell line are discussed.

2. Materials and methods

Phosvitin, casein and hemin were obtained from Sigma Laboratories and [γ^{32}P]ATP from the Radiochemical Center (Amersham, England). Tilorone was a generous gift from Merrel Laboratories. Quinacrine came from Specia Laboratories.

Hemin was prepared as described by Woodward et al. [12].

2.1. *Preparation of non-histone proteins of chromatin (NHC)*

Male Wag rats were sacrificed by decapitation after 18 h fasting. The livers were quickly removed and the nuclei were prepared according to Aaronson and Blobel [13]. Their purity was checked by phase contrast microscopy and in some cases by electron microscopy. NHC were prepared by a modification of the technique of Wang [14,15]. After removal of nuclear soluble proteins and most of nuclear particles by several washings with a solution containing 0.05 M NaCl, 0.05 M NaF, 0.05 M Tris, pH 7.0 (2 ml/g liver), chromatin was extracted with 2 M NaCl (1 ml/g liver) by homogenizing the washed nuclei in a Potter homogenizer and centrifuging 30 min at 10 000 × g. The dissociated chromatin was dialyzed overnight against 13 vol. 0.15 M NaCl, then centrifuged 15 min at 10 000 × g. The supernatant contained the non histone proteins.

2.2. *Determination of protein kinase activity*

Protein kinase activity was measured in the following standard assay mixture: 50 mM Tris, 2 mM EDTA, 0.5 mg protein substrate, 40–60 μg NHC and 0.1 mM [γ^{32}P]ATP. When the activity was measured on phosvitin, the $MgCl_2$ concentration was 2 mM and the pH adjusted to 7.5 [16]. On casein the $MgCl_2$ concentration was 20 mM and the pH 8.0 [16]. On NHC the $MgCl_2$ concentration was 10 mM and the pH 8.0 [16]. The final volume of the assay mixture was 0.5 ml.

Table 1
Effect of hemin on phosphorylation of phosvitin and casein

Hemin concentration (μM)	cpm Incorporated/mg protein substrate, % inhibition			
	Incorporation into phosvitin (cpm/mg prot.)	(%)	Incorporation into casein (cpm/mg prot.)	(%)
0	7698		3240	
10	6306	18	3380	0
50	2200	72	800	75
100	1734	78	832	75

Protein kinase activity was determined as indicated in Materials and methods. Specific radioactivity of ATP was $2.3.10^5$ cpm/nmol. About 50 μg NHC was added to 0.5 ml of the reaction mixture. To determine incorporation of ^{32}P into phosvitin and casein, the radioactivity incorporated in NHC alone was subtracted

The reaction was initiated by adding ATP and was allowed to proceed for 10 min at 37°C; 50 μl aliquots were then applied on Whatman 3 MM disk filters of 25 mm diameter and immediately dipped into a beaker containing cold 10% trichloracetic acid and 5.10^{-3}M ATP. After washing with 10% trichloracetic acid, the disks were rinsed twice with a solution containing 5% trichloracetic acid, 10^{-2}M $Na_4P_2O_7$, 10^{-2}M Na_3PO_4 [4]; and after washing with acetone–alcohol (1/1) the disks were dried and counted by Cerenkov effect.

2.3. *Electrophoretic analysis of phosphorylated NHC*

After incubation, proteins were precipitated by TCA (10% final) washed with cold 10% TCA containing 5.10^{-3} M ATP dissolved in 0.5–1 ml Laemmli sample buffer [17] and dialyzed overnight against 500 ml of the same buffer.

Electrophoresis in the presence of SDS was then performed according to Laemmli [17] using a 10% acrylamide separation gel and was carried out at 110 V for 5 h. After staining with Coomassie Blue, gels were dehydrated, dried, and autoradiography was performed by applying a Royal blue Kodak film on the dried gel for 4–7 days.

3. Results and discussion

The effect of hemin on several protein kinase activities of NHC is shown on table 1. It can be seen that the activities on phosvitin and casein are strongly inhibited by hemin. In the conditions of the test, at 100 μM hemin, more than 80% of the activity of the kinases is inhibited.

Protein kinase activities are also inhibited with non-histone proteins as substrates. The degree of inhibition is less than for phosvitin and casein: 60% at 100 μM hemin, but for 500 μM it attains 80% (table 2).

Table 2
Effect of hemin on phosphorylation of non-histone proteins of chromatin (NHC)

Hemin concentration (μM)	cpm Incorporated/mg NHC	% Inhibition
0	27 540	0
10	23 760	14
50	15 280	44
100	11 590	58
500	1540	95

Protein kinase activity was determined as indicated in Materials and methods. Specific radioactivity of ATP was $2.6.10^5$ cpm/nmol. The reaction mixture (0.5 ml) contained 0.5 mg NHC

As more enzymes are present in these autophosphorylation conditions (500 μM instead of 50 μg), this result is not unexpected if hemin combines with the kinases [18].

It seems, however, that at least one protein kinase activity is not inhibited. When electrophoresis of phosphorylated non-histone proteins followed by autoradiography is performed, two polypeptides approx. 65 000 daltons and 50 000 daltons, respectively, remain labeled even at 500 μM of hemin (fig.1).

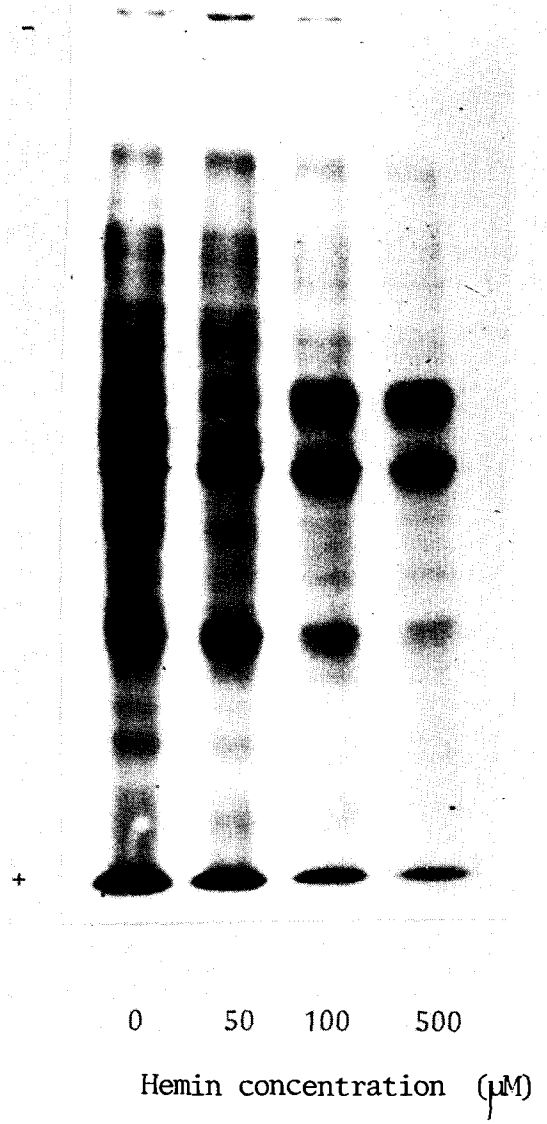

Fig.1. Autoradiographic patterns of phosphorylated NHC in the presence of various concentrations of hemin. 0.5 mg NHC in 0.5 ml were phosphorylated and submitted to SDS electrophoresis and autoradiography as indicated in Materials and methods.

The number and the specificity of the chromatin protein kinases is always under discussion, Dastugue et al. [16] have found at least 6 different activities whereas Kish et al. [19] have reported more than 12 different activities. According to Dastugue et al. [16] and more recently, to Dahmus [20], some of these kinases are present in nuclei and cytoplasm. Our results are not against this assumption, as hemin inhibit kinases associated with chromatin as well as with microsomes, both groups phosphorylating preferentially phosvitin. But at least one kinase of chromatin is different as it is not inhibited by hemin.

We have recently shown [4] that the microsomal protein kinase inhibition by hemin is reversed by the interferon inducer tilorone (2,7-bis, 2-diethyl amino ethoxy fluoren-9-one dihydrochloride). Similar results are obtained with NHC protein kinases as shown on table 3 where it can be seen that, at 10^{-4}M, tilorone re-establishes 75–80% of the activity. An other interferon inducer, quinacrine, has the same effect.

The site of action of interferon inducers is not known. It may be at a transcriptional or a translation level [21]. It is not known if hemin is present in the nucleus. But the inhibition by hemin and the reversion of this inhibition by tilorone and quinacrine may be a model for the regulation of some of the chromatin protein kinases by effectors, this by analogy with

Table 3
Effect of tilorone and quinacrine on phosphorylation of phosvitin

Tilorone (M)	– Hemin	+ Hemin (50 μM)
10^{-4}	9600	7600
10^{-5}		3340
10^{-6}		2240
0	9900	1800
Quinacrine		
3.10^{-4}	7600	7280
0	9600	1800

Same conditions as in table 1 were used. Specific radioactivity of ATP was $2.7.10^{-5}$ cpm/nmol.

cyclic AMP which regulates the activity of the cyclic AMP dependent protein kinases.

The role of these phosphorylations is not established, but there is a close correlation between the level of phosphorylation of the non-histone proteins of chromatin and the level of RNA synthesis [8,9]. This may occur through mechanisms which are certainly complex, as one would expect according to the heterogeneity of these proteins.

It is known that during the inactivation of the nuclei of the red cell line, the degree of phosphorylation of the non-histone proteins decreases strongly [22]. It is interesting to note that the only unnucleated cells or cells with inactive nuclei are the red cells.

There is a correlation between the level of hemoglobin synthesized and present in the cell during the erythroblast maturation and the degree of inactivation of the nucleus of red cell (Stohlman [23], Arnstein [24]). Stohlman has made the hypothesis that the cessation of nucleic acid synthesis and cell division relate to the cytoplasmic hemoglobin concentration, which would directly affect the intranuclear concentration. The presence of hemoglobin in the nucleus of nucleated red cells remains an open question. As high levels of hemin are present in the cytoplasm, it can be postulated that some molecules of hemin penetrate into the nucleus, possibly trapped during mitosis, and can thus inhibit the chromatin protein kinases.

Further work is necessary to test this hypothesis, which does not exclude other mechanisms like the appearance of histone H5. Hemin incubated with purified nuclei inhibits most of the nuclear phosphorylation (unpublished results). The activities of the endogenous RNA polymerases after incubation of nuclei with hemin are currently being studied in our laboratory.

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