Vol. 114, No. 2, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 29, 1983 Pages 612-619

THE EFFECT OF HEMIN AND OF ALLYL ISOPROPYL ACETAMIDE ON PROTEIN SYNTHESIS IN RAT HEPATOCYTES

Remi FAGARD* and Christiane GUGUEN-GUILLOUZO**

* Institut de Pathologie et Biologie Cellulaires et Moléculaires
INSERM U.15 - Université Paris V - CNRS L.A. 85
24, rue du Faubourg Saint-Jacques, 75014 PARIS (France)

** Unité de Recherches Hépatologiques U.49 de l'INSERM
Hôpital Pontchaillou - 35011 RENNES Cedex (France)

Received June 12, 1983

SUMMARY : Protein synthesis was measured in incubated hepatocytes. While hemin brings about a slight stimulation, allyl isopropyl acetamide (a compound that destroys the heme bound to cytochrome P450) inhibits protein synthesis by a mechanism that appears to result exclusively from depletion of cytoplasmic heme. Indications that in hepatocytes, as in reticulocytes, protein synthesis may be in part regulated by heme at the level of initiation are : i) that inhibition is accompanied by polysome breakdown ; ii) that the protein synthesis inhibitor already isolated from rat liver, is hemin reversible iii) that hepatocyte extracts contain a Mr 38,000 phosphoprotein which comigrates with the Mr 38,000 subunit of rabbit initiation factor 2 and iv) that the phosphorylation of both of these subunits is inhibited by hemin.

In reticulocytes and reticulocyte lysates protein synthesis is regulated by heme (1). In the absence of heme, synthesis proceeds for several minutes, then stops abruptly. Activation of a heme regulated inhibitor (HRI) during heme deficiency has been demonstrated (2,3). It appears to cause inhibition of protein synthesis by specific phosphorylation of the α subunit of eukaryotic initiation factor 2 (eIF-2α) (1,4-7, 10,11). However, in cells other than reticulocytes, regulation of protein synthesis by heme has not been clearly demonstrated. In Ehrlich ascites cells, inhibition of protein synthesis occured during nutrient deprivation (12). In rat liver, an inhibitor of protein synthesis has been isolated (13). We investigated the effect of hemin on protein synthesis in rat hepatocytes both directly by adding it to the incubation medium of cell suspensions, and indirectly by adding allyl isopropyl acetamide, a compound that destroys heme bound to cytochrome P450 and consequently depletes the cytoplasmic heme pool (14,15,20).

0006-291X/83 $1.50

MATERIALS AND METHODS

Preparation and incubation of hepatocytes : Hepatocytes were freshly isolated from male rats (150-200 g) (Wistar) by perfusion of the liver with a collagenase solution according to Seglen (16). Cell viability was determined by the measurement of trypan blue exclusion, albumin secretion and lacticodehydrogenase release. Hepatocytes (4×10^6) were incubated (17) in : glucose : 1 mg/ml ; $NaHCO_3$: 0.4 mg/ml ; amino-acids cocktail (except leucine) 2 mM ; leucine 0.5 mM ; ^3H-leucine (40 Ci/mmole, NEN) : 400 µCi per incubation ; dialysed Fetal calf serum : 5 %. Incubations (2 ml) were placed in a shaker at room temperature [shown to give optimal cell survival]. Aliquots (0.5 ml) were taken at intervals and processed as described in the legends.

Preparation and incubation of rabbit reticulocytes and rabbit reticulocyte lysates : Reticulocytes were prepared and incubated as described (17,18). Preparation and incubation of reticulocyte lysates was performed as previously described (18,19).

Phosphorylation assays and polyacrylamide gel electrophoresis : Incubations (20 µl) contained [$^{32}P\gamma$]-ATP (5000 Ci/mmole, Amersham) 30 µCi ; tris-HCl 20 mM pH 7.6 ; KCl 80 mM ; Mg $(OAc)_2$ 2 mM ; high speed supernatant (containing L.I) (2 µg) and ribosomal salt wash (containing eIF-2) (1 µg). Incubations were performed at 30° for 5 minutes and stopped by the addition of an equal amount of sample buffer (5), after 5 minutes at 95° the samples were separated on SDS acrylamide slab gels (10 % acrylamide) as previously described (5). After staining and destaining autoradiograms of the dried gels were obtained on XOMAT-AR films (Kodak).

Measurement of tryptophane pyrrolase activity : The method of Badawy and Morgan (21) was used. Hepatocyte homogenates (3 ml) from 40×10^6 cells were supplemented with tryptophane 25 mM in 50 mM phosphate pH 7 to a final volume of 12 ml and placed at 37° under O_2 in a shaker. At time intervals the incubations were stopped by the addition of 0.66 ml TCA 14 %, centrifuged and 1 ml of the supernatant supplemented with 0.6 ml of 0.06 N NaOH. The formation of kinurenin due to the activity of the enzyme was detected by measuring the optical density at 365 nm in a Zeiss spectrophotometer. Since this determination was comparative arbitrary units were used.

Preparation of rat liver extracts : After perfusion with buffer 1 (50 mM tris-HCl pH 7.5 ; 50 mM KCl ; 5 mM $Mg(OAc)_2$; 5 mM GSH ; 250 mM Sucrose) livers were removed and placed in the same medium at 0°, sliced and homogenized. The post mitochondrial supernatant was obtained and centrifuged for 120 mn at 150,000g at 4° in a Beckman centrifuge. Crude liver inhibitor (L.I) was obtained as follows : the post-microsomal supernatant was slowly adjusted to pH 5.1 at 0°, after centrifugation the pellet was resuspended in buffer 2 (50 mM tris-HCl pH 7.6 ; 50 mM KCl ; 5 mM Mg $(OAc)_2$; 10 % glycerol) and dialysed against the same buffer. Ammonium sulfate was then added to 25 %, the pellet discarded and the supernatant slowly adjusted to 42 %. The pellet was resuspended and dialysed against buffer 2. Small aliquots of this preparation called crude L.I. were kept in liquid nitrogen and contained 2 µg/µl protein. Ribosomal salt wash (RSW) was obtained by resuspension of the microsomal pellet in buffer 2 and slow addition of 0.55 M KCl at 0° with gentle stirring for 20 minutes. After centrifugation the supernatant was dialysed against buffer 2 and adjusted to 45 % ammonium sulfate ; the pellet was resuspended in buffer 2 dialysed and kept in liquid nitrogen.

Polysome profiles : The method described by Dickson and Pogson (23) was used with minor modifications.

Partial proteolysis mappings : The method described by Cleveland et al. (22) was used with minor modifications, substilisin (Sigma) (0.5 µg) was used with 0.2 mM CaCl2.

Allyl Isopropyl Acetamide was a generous gift from Hoffmann-Laroche (Basel). Rabbit eIF-2 was a generous gift of Dr Hans Trachsel (Basel).

RESULTS AND DISCUSSION

Effects of hemin and allyl isopropyl acetamide on hepatocyte protein synthesis:

After hemin was added to the incubation medium containing freshly isolated hepatocytes, a slight stimulation of protein synthesis was detectable (figure 1). In contrast, when allyl isopropyl acetamide (AIA) was added to the incubation medium, concentration dependent inhibition of protein synthesis was observed as shown in figure 1. To see if inhibition of protein synthesis by AIA was the result of heme depletion, an excess of hemin was added to the AIA (400 µg/ml) supplemented incubation. One hour after the addition of 200 µM of hemin, the recovery of protein synthesis ranged from 50 to 100 %. Lower concentrations of hemin (25 to 50 µM) failed to reverse AIA inhibition. The intracellular pool of free heme was measured by determining tryptophane pyrrolase (TP) activity ; this enzyme uses heme as cofactor and its activity reflects the size of the free heme pool (14,21). It has been shown previously that

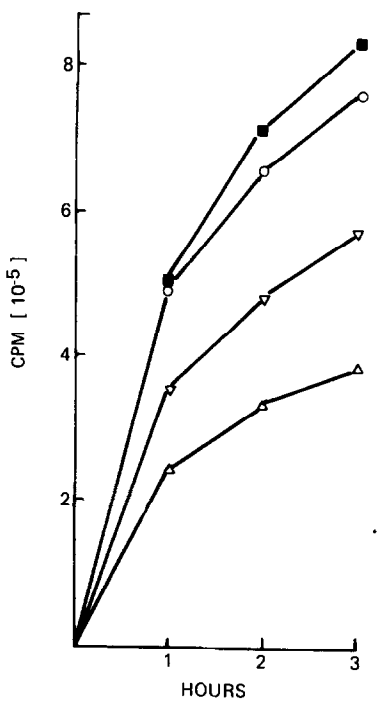

Figure 1. Effect of hemin and of AIA on protein synthesis in hepatocytes. Incubations (1.8 ml) containing 4 x 10^6 freshly prepared hepatocytes were at room temperature in a shaker. Aliquots (0.5 ml) were taken at intervals and washed in phosphate 0.1 M pH 7.4, NaF 50 mM, EDTA 8 mM. The pelleted cells were frozen and thawed, lysates were washed in hot and cold TCA 5 %, after resuspension in formic acid protein pellets were counted in a scintillation counter. No additions (○) ; AIA (200 µg/ml) (▽) ; AIA (400 µg/ml) (△) ; hemin (50 µM) (■).

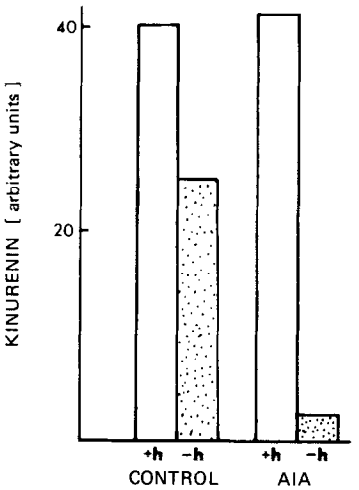

Figure 2. Tryptophane pyrrolase activity in lysates of hepatocytes incubated with AIA. The enzymatic activity was measured as described in methods. The whole lysates of control and treated cells were incubated at room temperature with constant shaking under oxygen. Since this experiment was intended to compare normal to AIA treated cells results are expressed in arbitrary units of kinurenin formed in the absence or in the presence of 2 μM hemin. Cells were treated with 400 μg/ml AIA or saline (control) for 1 hour.

TP activity is maximally depleted 30 minutes after administration of AIA to rats (21). We studied TP activity in rat hepatocytes after one hour of incubation. The basal activity of TP was 25 units. When hemin was added, the activity increased to 40 units. Lysates from AIA treated hepatocytes showed a strong decrease in TP activity (5 units). However, the same experiment carried out in the presence of hemin, yielded TP activity that was identical with the control (figure 2). These results indicate that AIA treated hepatocytes have a depleted heme pool. Therefore, the inhibition of protein synthesis observed with AIA appears to result from depletion of the free heme pool and not from other possible mechanisms such as the accumulation of green pigments (14,15). Neither microscope examination of the cells, nor measurement of LDH release showed a difference between treated and control cells suggesting that no cell lysis occured. In addition, albumin secretion was identical (results not shown). However, the possibility remained that AIA could specifically block the polysomal machinery. To check this point, various concentrations of AIA were added to rabbit reticulocyte lysates. No inhibition of protein synthesis was observed with the concentrations used (figure 3a). In addition, no inhibition of protein synthesis occured when AIA was added to rabbit reticulocytes, which do not contain cyto-

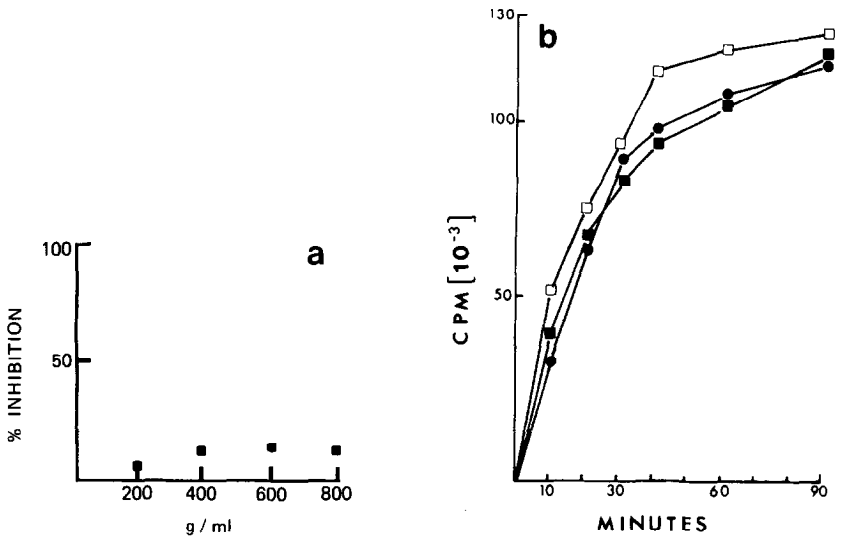

Figure 3a. Effect of AIA on protein synthesis in reticulocyte lysates. Incubations (20 µl) containing 50 % lysate were supplemented with various concentrations of AIA and the incorporation of ^{14}C-leucine measured on aliquots (5 µl) after 20 minutes of incubation at 30°. The incorporation in the control incubation was 14 000 cpm. Inhibition by heme deficiency was 55 %. Results are expressed in % inhibition.

Figure 3b. Effect of AIA on protein synthesis in incubated reticulocytes. Rabbit reticulocytes were incubated as described (17,18). Incubations (1.5 ml) were performed at 30° with gentle shaking and contained: no additions (●) ; AIA (400 µg/ml) (■) ; hemin (100 µM) (□). Aliquots (50 µl) were removed, placed on paper filters and processed as described (18).

chrome P450 (figure 3b), addition of hemin resulted in a slight stimulation of protein synthesis. These results confirm the hypothesis that AIA can only destroy the heme on cytochrome P450 and causes rapid depletion of the free heme pool of the hepatocyte cytoplasm.

Level of inhibition of protein synthesis :

Depletion of cytoplasmic heme by AIA is correlated with the inhibition of protein synthesis. The polysome profiles of AIA treated hepatocytes showed a breakdown of heavy polysomes (figure 4) and an increase of the 80 S peak. These results suggest that in isolated hepatocytes AIA induces polysome disaggregation by inhibiting re-initiation. In reticulocytes, this phenomenon has been shown to result from the inhibition of protein synthesis (24). Polysome disaggregation following AIA treatment is solid evidence that protein synthesis is blocked.

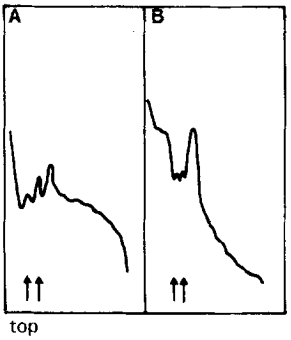

Figure 4. Modification of the polysomal profile by AIA. Polysomes (1.4 A260 units) were separated on 15-50 % sucrose gradients : polysomes from hepatocytes (40 x 10^6 per incubation) treated with AIA (400 µg/ml) for 1 hour (B). Polysomes from control hepatocytes (A). The 2 arrows indicate the position of the 40S and 60S subunits, determined by analysing polysomes treated with 100 mM EDTA.

Delaunay et al. (13) described an inhibitor of protein synthesis (LI) which they showed to be an eIF-2α Kinase that inhibits protein synthesis in reticulocyte lysates. The experiment shown in figure 5 demonstrates that incubation of LI

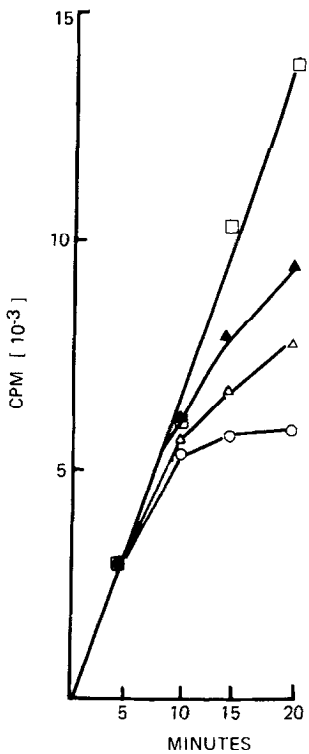

Figure 5. Hemin reversibility of L.I. as tested in a reticulocyte lysate. Incubations (25 µl) were as described in methods, aliquots (5 µl) were removed and the radioactivity measured. The liver inhibitor (L.I.) (4 µg) was added at the beginning of the incubation (△) ; L.I. (4 µg) was incubated for 1 minute at 30° with hemin (20 µM) before addition (▲). All incubations contained hemin (20 µM) except the minus hemin control (□). Hemin supplemented control (○).

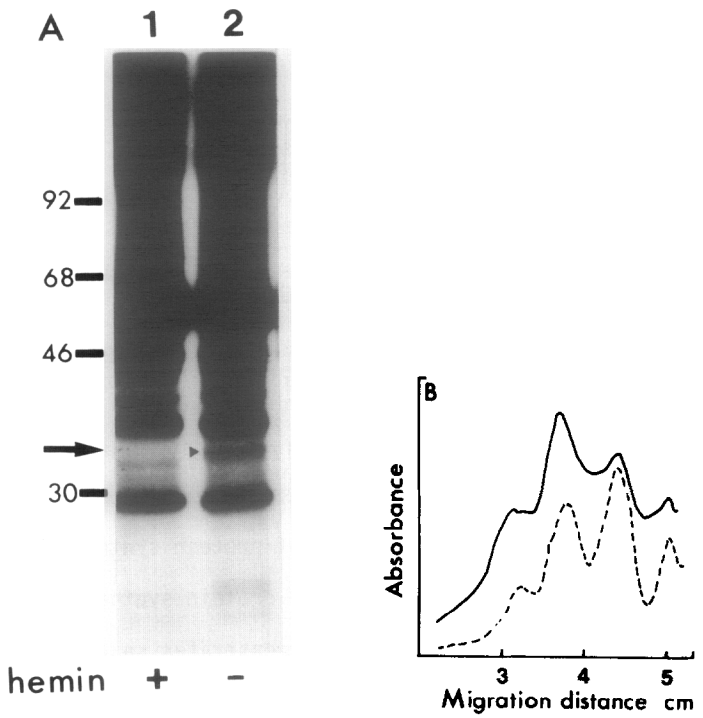

Figure 6. Effect of hemin on the phosphorylation profile of liver preparations.

A : Crude L.I. (2 µg) and R.S.W. (containing eIF-2) (1 µg) were incubated with [γ-^{32}P] ATP and hemin (20 µM) (slot 1) or no hemin (slot 2). The arrow indicates the migration of the Mr 38,000 subunit of rabbit eIF-2. The figure is an autoradiogram.

B : Scan of a partial proteolysis mapping of phosphorylated rabbit eIF-2α (——) and the 35K phosphoprotein of rat R.S.W. (---). The phosphorylated bands were excised from SDS gels swollen and re-run on gel with 0.5 µg protease (subtilisin), scans of the autoradiogram were obtained on a Gelman scanner.

with hemin (20 µM) prior to the addition to the lysate resulted in a significant reversal of inhibition. Therefore, LI appears to be both an eIF-2α kinase and a hemin reversible inhibitor of protein synthesis and thus ressembles closely the reticulocyte inhibitor (HRI) (2,3). The possibility that phosphorylated eIF-2α was present in rat liver cells was also investigated. On SDS-acrylamide gels, a phosphoprotein was detected whose phosphorylation was abolished by hemin (20 µM) (figure 6a). The following results indicate that this phosphoprotein may be eIF-2α. It co-migrates with the α subunit of purified rabbit eIF-2 and the partial proteolysis mappings of the two polypeptides are very similar (figure 6b).

In conclusion, hemin slightly stimulates protein synthesis in hepatocytes. This may be due to the optimal concentration of heme present in these cells. The inhibition by AIA appears to result from the depletion of the endogenous free heme pool. Our data suggest that in hepatocytes as in reticulocytes, protein synthesis may be regulated by hemin.

ACKNOWLEDGMENTS

This work was financially supported by the Institut National de la Santé et de la Recherche Médicale and by the Centre National de la Recherche Scientifique.

REFERENCES

1. London, I.M., Ernst, R., Fagard R., Leroux, A., Levin, D.H. and Petryshyn R. (1981) Cold Spring Harbor Conferences on Cell Proliferation 8 Protein phosphorylation 941-957.
2. Maxwell C.R. and Rabinovitz, M. (1969) Biochem. Biophys. Res. Commun. 35 79-85.
3. Gross, M. and Rabinovitz, M. (1973) Biochem. Biophys. Res. Commun. 50, 832-838.
4. Farrell, P.J., Balkow, K., Hunt, T., Jackson, R.J. and Trachsel, H. (1977) Cell 11, 187-200.
5. Levin, D.H., Ranu, R.S., Ernst V. and London, I.M. (1976) Proc. Natl. Acad. Sci. USA 73, 3112-3115.
6. Kramer, G., Cimadevilla M. and Hardesty, B. (1976) Proc. Natl. Acad. Sci. USA 73, 3078-3082.
7. Gross, M. and Mendelewski, J. (1977) Biochem. Biophys. Res. Commun. 74, 559-569.
8. Jagus, R., and Safer, B. (1981) J. Biol. Chem., 256, 1317-1323.
9. Kaplansky, D.A., Kwan, A. and Gross, M. (1982) J. Biol. Chem. 257, 5722-5729.
10. Hunt T. (1980) in : Recently discovered systems of enzyme regulation by reversible phosphorylation (P. Cohen ed.) Elsevier/North Holland Biochemical Press pp 175-202.
11. Jagus, R., Anderson, W.F. and Safer, B. (1980) in Prog. Nucl. Acid. Res. Mol. Biol. Vol. 25 Acad. Press Inc. pp 127-185.
12. Van Venrooij, W.J.W., Henshaw, E.C. and Hirsch, C.A. (1972) Biochim. Biophys. Acta 259, 127-137.
13. Delaunay, J., Ranu, R.S., Ernst, V. and London, I.M. (1977) Proc. Natl. Acad. Sci. USA 74, 2264-2268.
14. De Matteis, F. (1971) Biochem. J. 124, 767-777.
15. Granick, S. and Beale, S.I. (1978) Advances in Enzymology 46, 33-203.
16. Seglen, P.O. (1973) Exp. Cell Res. 76, 25-30.
17. Bitte, L. and Kabat, D. (1979) Meth. in Enzymology 30, 563-601.
18. Leroux, A., and London, I.M. (1982) Proc. Natl. Acad. Sci. USA 79, 2147-2151.
19. Hunt, T., Vanderhoff, G. and London, I.M. (1972) J. Mol. Biol. 66, 471-481.
20. Grandchamp, B., Bissell, D.M., Licko, V. and Schmid, R. (1981) J. Biol. Chem. 256, 11677-11683.
21. Badawy, A.A.B., and Morgan, C.J. (1980) Biochem. J. 186, 763-772.
22. Cleveland, D.W., Fischer, S.G., Kirschner, M.W. and Laemmli, U.K. (1977) J. Biol. Chem. 252, 1102-1106.
23. Dickson, A.J. and Pogson, C.I. (1980) Biochem. J. 186, 35-45.
24. Grayzel, A.I., Hörchner, P. and London, I.M. (1966) Proc. Natl. Acad. Sci. USA, 55, 650-655.