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THE INFLUENCE OF PROPRANOLOL ON THE CONCENTRATION OF HEME AND ON THE ACTIVITY OF δ-AMINOLEVULINATE SYNTHASE IN MONOLAYERS OF CHICK EMBRYO LIVER CELLS

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Abstract—The addition of propranolol to monolayers of chick embryo liver cells caused a rapid increase in cellular heme, followed by an equally rapid decrease. Subsequently the concentration of heme rose at a relatively slower rate. About 10 hr after addition of propranolol to the medium a plateau level was reached at ± 35% above control values. Changes in the activity of δ-aminolevulinate synthase (ALAS) were negatively correlated with those of cellular heme. Cycloheximide prevented the above phenomenon. ALAS activity was not clearly correlated with the rapid, partial inhibition of protein synthesis, caused by propranolol.

These observations are related to the beneficial influence of administration of hemin or of propranolol to patients with acute attacks of hepatic porphyria.

Until a decade ago acute attacks of hepatic porphyrias were treated with large amounts of glucose or other carbohydrates [1–4], with partial success only. In 1971 intravenous administration of heme was introduced by Bonkowsky *et al.* [5]. Further experience showed this treatment to be effective in most patients, especially if initiated before symptoms of paralysis appeared [6, 7]. In 1972 and later it was shown by us that large doses of propranolol have a markedly beneficial effect on acute attacks [8–10]. Even in a comatose, quadriplegic patient the administration of propranolol completely reversed the pathological processes [11].

Since the clinical and biochemical effects of propranolol are very similar to those observed after administration of heme, this study was undertaken in order to investigate the effect of propranolol on cellular heme and on the activity of δ-aminolevulinate synthase (ALAS) in monolayer cultures of chick embryo liver cells.

MATERIALS AND METHODS

Cultures of chick embryo liver cells were prepared according to Sassa and Kappas [12], but the final pellets of cells were resuspended in one hundred times their volume of modified Ham's F12 with hormones. Monolayers were prepared in 3.5 cm diameter tissue culture dishes (Nunclon, Roskilde, Denmark), 2 ml of suspension in each dish. The cultures were incubated for 18 hr in humidified air, 95%, and CO_2, 5%, at 37°, after which the medium was replaced and the various additives or their vehicles were added. At the appropriate times the medium was removed and the cells were scraped off with 1 ml of distilled water.

Cellular heme was determined by the method of Morrison [13] as adapted by Sassa and Kappas [12]. The activity of ALAS in homogenates of the cells was determined according to Method II of Sinclair and Granick [14]. Protein synthesis was determined by measuring the incorporation of [^{14}C]leucine into proteins as described previously [15]. Protein was determined by the method of Lowry *et al.* [16] with bovine serum albumin as standard. Modified Ham's F12 medium was prepared by the Laboratory of Public Health, Jaffa, Israel. Collagenase and hyaluronidase were obtained from The Grand Island Biological Company, Grand Island, NY. DL-Propranolol was kindly supplied by Mgr. G. Keizman, Abic Ltd., Ramat Gan, Israel. D-Propranolol was a gift from Dr. H. A. Johnston, Imperial Chemical Industries, Macclesfield, U.K. All other chemicals were of reagent grade.

RESULTS

Twenty-four hours after replacement of the medium, i.e. 42 hr after preparing the cultures, the concentration of heme in the liver cells was found to be 381 ± 60 pmole/mg protein. Both DL- and D-propranolol increased the heme content of the cells to approximately the same extent. The most marked effect, 30–40% increase, was obtained with both DL- and D-propranolol at a concentration of 30 μg/ml (Table 1).

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Table 1. The influence of propranolol on the concentration of heme in monolayers of chick embryo liver cells

Concentration of propranolol (μg/ml medium)	Per cent change in the concentration of cellular heme	
	DL-Propranolol	D-Propranolol
10	7.5 ± 5.5	6.5 ± 2.5
20	10.0 ± 6.0	16.0 ± 5.0
30	40.0 ± 5.5	31.5 ± 1.5
40	13.0 ± 10.5	15.5 ± 2.5
60	11.0 ± 20.0	17.0 ± 3.0

Monolayers of chick embryo liver cells were prepared as described in Methods. After 18 hr incubation the medium was replaced by fresh medium. At this time DL- or D-propranolol, dissolved in 0.9% NaCl, was added to the medium and NaCl only to control cultures. After a further incubation for 24 hr cellular heme and protein were determined. The results are expressed as per cent change in the concentration of cellular heme calculated on the basis of control cultures without propranolol which were regarded as 100%.

The values shown are the averages and standard deviations of the results obtained from 10 dishes in 5 separate experiments for DL-propranolol and from 6 dishes in 3 separate experiments for D-propranolol.

In order to further examine the influence of propranolol on cellular heme and its inter-relations with ALAS activity and over all protein synthesis the time course of the effects of propranolol was determined. Since it was shown by us previously that in this system both DL- and D-propranolol inhibit ALAS activity and protein synthesis to approximately the same extent [15, 17], only DL-propranolol was used. The results of these experiments are shown in Fig. 1.

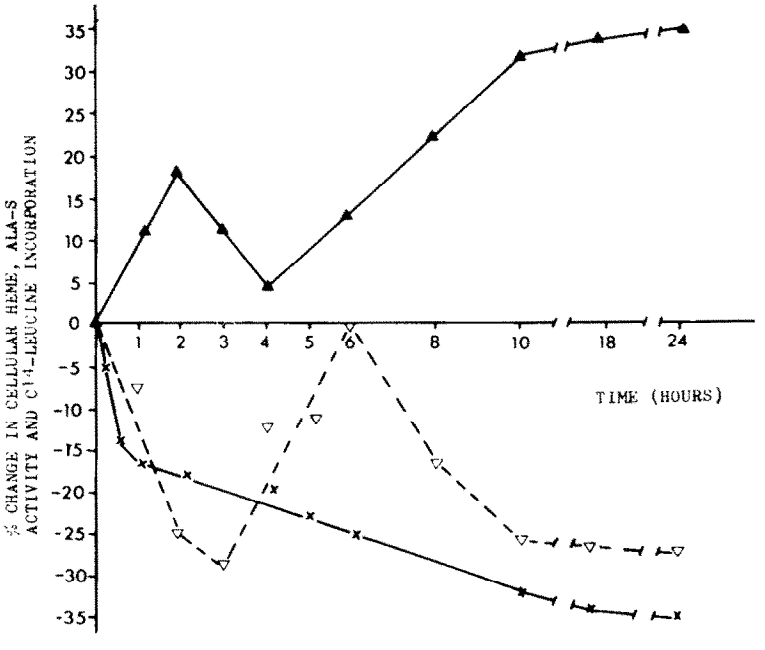

Fig. 1. The influence of DL-propranolol on the concentration of heme, ALAS activity and incorporation of [^{14}C]leucine into proteins. Monolayers of chick embryo liver cells were prepared as described in Methods. After 18 hr incubation the medium was replaced and DL-propranolol, 30 μg/ml medium, was added. At each time-point indicated heme, ALAS activity, [^{14}C]leucine incorporation and proteins were determined in duplicate dishes. For details of the determinations see Methods. The percentile changes indicated are the averages of 3–4 separate experiments. Differences in percentages between these experiments were less than 10%. Each value is based upon its control culture, examined at the same time, being regarded as 100%. The concentration of heme in the control cultures at time zero was 352.0 ± 52.9 pmole/mg protein. The activity of ALAS at zero time was 0.72 ± 0.05 nmole ALA/mg protein/30 min and the incorporation of [^{14}C]leucine was 3773 ± 243 dpm/mg protein/30 min. ▲ = heme; × = [^{14}C]leucine incorporation; ▽ = ALAS.

Table 2. Effect of cycloheximide on ALAS activity of liver cells treated with propranolol

Time from addition of cycloheximide (hr)	ALA nmole/mg protein/30 min	
	Control	DL-Propranolol-treated
0	0.83 ± 0.13	0.62 ± 0.09
1	0.44 ± 0.02	0.58 ± 0.07
2	0.24 ± 0.06	0.31 ± 0.07

Monolayers of chick embryo liver cells were prepared as described in Methods. After 18 hr incubation the medium was replaced by fresh medium. At this time DL-propranolol, 30 μg/ml, was added to test cultures while 0.9% NaCl was added to control cultures. After 3 hr incubation cycloheximide, 250 μg/ml, was added both to control and to propranolol treated cultures. ALAS activity was determined at this time (zero time) and 1 and 2 hr after addition of the cycloheximide. Each value represents the average and standard deviation of 4 determinations.

It was observed that addition of propranolol, 30 μg/ml medium, caused a rapid elevation of cellular heme with a peak at 2 hr from zero time. This was followed by a decline during the next 2 hr, after which the concentration of heme rose steadily to a plateau level which was about 35% above control cultures without propranolol. It should be noted that the concentration of heme in the control cultures declined during the 24 hr of the experiment, from 352.0 ± 52.9 pmole/mg protein at zero time to 281.0 ± 32.2 pmole/mg protein at 24 hr. In additional experiments the plateau level of heme concentration did not change during an additional 36 hr (not shown).

In contrast to the initial increase in the concentration of heme the activity of ALAS decreased after addition of propranolol. The lowest point, 28% below control values, was reached about 3 hr after addition of propranolol. Subsequently this activity rose, reaching a zenith at the level of the control cultures a short time after the heme concentration reached its nadir. This was followed by a second decline of ALAS to a plateau at about 30% below that of control cultures. The ALAS activity of the control cultures did not change appreciably during the 24 hr of the experiment.

The incorporation of [^{14}C]leucine into proteins dropped precipitously during the first 60 min after the addition of propranolol. Subsequently protein synthesis continued to decline slowly to 35% below control values after 24 hr.

In another series of experiments the influence of cycloheximide on the effects of propranolol on ALAS activity and cellular heme was examined. In this system cycloheximide, 250 μg/ml medium, inhibits the incorporation of [^{14}C]leucine into proteins by 95% [15]. Cycloheximide was added to cultures 3 hr after addition of propranolol and ALAS activity was determined at this time (zero time) and after 1 and 2 hr of further incubation. At the time of addition of cycloheximide the activity of ALAS in the cultures treated with propranolol for 3 hr was 25% lower than that in control cultures (Table 2).

As shown in Table 2 the addition of cycloheximide to the control cultures caused a rapid decrease of ALAS activity with a half-life of about 60 min. Addition of cycloheximide to the cultures previously incubated for 3 hr with propranolol not only prevented the increase of ALAS activity observed when no cycloheximide was present but caused it to decrease. Two hours after addition of the cycloheximide this activity was 40% less than that of

Table 3. Effect of DL-propranolol on the heme content of liver cells treated with cycloheximide

Time from addition of cycloheximide (hr)	Heme pmole/mg protein	
	Control	DL-Propranolol-treated
0	274	274
1	263	263
2	242	232
3	239	265
4	220	244
6	198	214

Monolayers of chick embryo liver cells were prepared as described in Methods. After 18 hr incubation the medium was replaced by fresh medium. Cycloheximide, 250 μg/ml, was added to both control and test cultures. At the same time DL-propranolol, 30 μg/ml, was added to test cultures only. Cellular heme and proteins were measured at zero time and at various time points afterwards. Zero time = time of addition of DL-propranolol and cycloheximide.

The values are the average of 2 determinations in a representative experiment. The same pattern was observed in an additional experiment.

cultures with propranolol but without cycloheximide (not shown). The half-life of ALAS activity in the presence of both propranolol and cycloheximide is longer than that in the presence of cycloheximide only.

Addition of cycloheximide together with propranolol prevented the initial rapid rise in cellular heme observed when only propranolol was added to cultures. Concentration of cellular heme declined during the first 2 hr, increased during the next 1–2 hr and afterwards declined again. When only cycloheximide was added to the cultures the cellular heme concentration decreased gradually during the 6 hr period examined (Table 3). In control cultures without cycloheximide and/or propranolol the heme concentration decreased by 6% during the 6 hr of the experiment.

DISCUSSION

Propranolol has a markedly beneficial effect upon the clinical and biochemical symptoms of acute intermittent porphyria [10, 11], variegate porphyria [8, 9], and of coproporphyria [18]. In rats and in cultures of chick embryo liver cells propranolol partially inhibits the induction of ALAS by allyl-isopropylacetamide (AIA) and by 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) [17, 19, 20]. Subsequent investigations showed that propranolol inhibits the incorporation of amino acids into proteins [15] and of thymidine and uridine into nucleic acids [21]. It also inhibits the transport of α-aminoisobutyric acid [22] and the transport or phosphorylation of 2-deoxyglucose [23] as examined in monolayers of chick embryo liver cells. The extent of these inhibitions is about the same as that by which propranolol inhibits the induction of ALAS by AIA or DDC.

It was shown in this investigation that intracellular heme rises rapidly during the first 2 hr after addition of propranolol to the medium, followed by an equally rapid decline. Subsequently a steady increase is observed till a plateau level of about 33% above control values is reached.

The initial rapid rise is most probably not caused by a fast increase in heme synthesis since simultaneously the ALAS activity is reduced. It may, therefore, be assumed that during these first 2 hr the rate of heme catabolism decreases even more than that of heme synthesis. This could be the result of a decrease in the activity of heme oxygenase or other pathways of heme catabolism [24]. Following the initial increase in heme the secondary decrease could be caused by induction of heme oxygenase [25]. The final increased concentration of cellular heme in the presence of propranolol represents, probably, a new steady state in which both heme synthesis and catabolism are reduced. However, under these conditions heme may be partially inaccessible to heme oxygenase.

Cycloheximide prevented the initial increase in the concentration of cellular heme caused by propranolol. However, several hours after the simultaneous addition of cycloheximide and propranolol a slight increase in the concentration of heme was observed, compared to cultures with cycloheximide only. At present we are investigating these phenomena.

The initial rapid increase in cellular heme which is observed after addition of propranolol would, probably, increase its concentration in the 'free' or 'regulatory' heme pool. This would cause repression of ALAS synthesis, resulting in the observed initial decrease in ALAS activity, which lags slightly behind the increase in heme. The subsequent decrease in cellular heme explains the secondary rise in ALAS activity, which, indeed, is slightly delayed. This increase in ALAS activity is not caused by an activation process of pre-existing enzyme since it is abolished by the addition of cycloheximide. During the third phase, when cellular heme is elevated, ALAS activity is low. Figure 1 shows clearly that ALAS activity is inversely related to the concentration of intracellular heme and lags slightly behind the changes in the latter. This is exactly what would be expected to occur on the basis of the hypothesis of regulation of ALAS activity by the concentration of heme in the 'regulatory' heme pool, provided the changes in cellular heme reflect parallel changes in the 'regulatory' pool. A previously made observation was again confirmed during this investigation, i.e. that addition of propranolol seems to prolong the half-life of ALAS (Table 2). This phenomenon is being further investigated.

In a previous investigation we reported that propranolol has a marked inhibitory effect on incorporation of [^{14}C]leucine into proteins. It was now shown that this inhibition is apparent within 15 min after addition of propranolol to the culture medium. It is of interest to note that during the first 6–8 hr after addition of propranolol the changes in ALAS activity are not parallel to the curve of inhibition of incorporation of [^{14}C]leucine into protein. Specifically, during the period between 2 and 4 hr after addition of propranolol to the medium, ALAS activity increases while protein synthesis remains strongly inhibited. Our previous explanation of the decrease in ALAS activity caused by propranolol as being due to a quantitatively similar decrease in protein synthesis [15, 22] may, therefore, be only partially correct.

The observations described in this report indicate that the overall effect of propranolol is to increase cellular heme in the system of chick embryo liver cells.

There are two main hypotheses, purporting to explain the neurological manifestations observed during acute attacks of hepatic porphyria and the biochemical basis of the amelioration of the clinical symptoms following heme administration. According to Watson and coworkers [6, 26] a deficit in intracellular heme during acute attacks causes neuronal damage and administration of heme replenishes the heme stores. According to other investigators [27] the neuronal malfunction is possibly caused by the high blood and tissue levels of δ-aminolevulinic acid (ALA). ALA was shown to compete with γ-aminobutyric acid in binding to receptors in the central nervous system and to exert a partial agonistic action. Administration of heme leads to a decrease in the activity of ALAS and thus to a

reduction in the concentration of ALA, followed by clinical improvement.

The data reported here are in accordance with the hypothesis that the clinical symptoms of porphyria are caused by a decrease in intracellular heme. However, they do not refute the hypothesis that high concentrations of ALA are responsible for the malfunction of the nervous system since an increase in cellular heme leads to repression of ALAS synthesis and a decrease in ALA. Our observations may explain the similarity of the beneficial effects observed by administration of either heme or propranolol to patients during acute attacks and the biochemical effects of propranolol in experimental porphyria. The exact mechanism of action of propranolol on intracellular heme requires further investigation.

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