Regulation of neutral cholesterol esterase and acyl-CoA :cholesterol acyltransferase in the rat adrenal gland

David M. BEINS, Ross VINING and Santhirasegaram BALASUBRAMANIAM*
*Lipid Research Division, Medical Professorial Unit and Garvan Institute of Medical Research,
St. Vincent's Hospital, Darlinghurst, N.S.W. 2010, Australia*

(Received 21 August 1981/Accepted 29 October 1981)

The activities of neutral cholesterol esterase and acyl-CoA :cholesterol acyltransferase in rat adrenal gland were measured at various time intervals over 24 h. The activity of cholesterol esterase displayed diurnal rhythm, with a major peak at the onset of darkness coinciding with the peak in the diurnal rhythm of plasma corticosterone concentration. The activity of acyl-CoA :cholesterol acyltransferase also exhibited a characteristic diurnal rhythm, with the minimum activity occurring 3 h after the onset of darkness. The profile of the rhythm exhibited by the activity of the esterifying enzyme was similar to the mirror image of the pattern of diurnal rhythm in the activity of 3-hydroxy-3-methylglutaryl-CoA reductase. Microsomal non-esterified cholesterol showed a gradual decline with a significant decrease in concentration at the onset of darkness, thus suggesting that diurnal removal of cholesterol in the environment of the esterifying enzyme and hydroxymethylglutaryl-CoA reductase leads to such diurnal decrease or increase in the activities of these two enzymes. Acute administration of corticotropin led to a 3-fold increase in the activity of cholesterol esterase, a 50% decrease in the activity of acyl-CoA :cholesterol acyltransferase and a 2-fold increase in the activity of hydroxymethylglutaryl-CoA reductase. Corticotropin administration also resulted in a significant decrease in microsomal non-esterified cholesterol and increase in plasma corticosterone concentration. These observations suggest that corticotropin plays an important part in generating the diurnal rhythm in the activities of the three enzymes.

Cholesterol in the adrenal gland, whether derived from sources outside the gland or synthesized *in situ*, is either converted into steroid hormones and released into the blood circulation or is esterified and stored in the intracellular lipid droplets. The esterification is catalysed by the enzyme acyl-CoA :cholesterol acyltransferase (EC 2.3.1.26) bound to the endoplasmic-reticular membrane. The hydrolysis of the esterified cholesterol is catalysed by the cytoplasmic enzyme cholesterol esterase (EC 3.1.1.13). Under extreme conditions such as stress, high plasma concentrations of corticotropin or a substantial decrease in the plasma concentration of cholesterol, the activities of both these enzymes have been shown to be altered (Trzeciak & Boyd, 1973; Boyd & Trzeciak, 1973; Balasubramaniam *et al.*, 1977a).

Previous work (Balasubramaniam *et al.*, 1977b)

shows that the activity of the enzyme 3-hydroxy-3-methylglutaryl-CoA reductase (EC 1.1.1.34), which regulates cholesterol biosynthesis, varies diurnally in a characteristic manner in the adrenal gland. Moreover, it has been well established that the plasma corticosterone concentration in the rat exhibits classical diurnal rhythm. These findings raise the possibility that such diurnal variation could also be demonstrable in the activities of cholesterol esterase and acyl-CoA :cholesterol acyltransferase.

Thus, to gain further insight into the extent to which these two enzymes influence adrenal cholesterol metabolism and plasma corticosterone concentration, we have measured the changes in the activities of these enzymes over 24 h consisting of equal periods of dark and light cycles. Moreover, to assess the role of corticotropin in the regulation of these enzymes, we have also attempted to correlate the changes in the activities of these two enzymes and of hydroxymethylglutaryl-CoA reductase with

* To whom requests for reprints should be addressed.

changes in the adrenal cholesterol concentration and plasma corticosterone concentration after administration of corticotropin.

Materials and methods

Materials

[1 α ,2 α (n)-³H]Cholesteryl oleate (54.4 Ci/mmol) and [4-¹⁴C]cholesteryl oleate (58.4 mCi/mmol) were synthesized by using the micro-oleoylation method of Goodman (1969). Synacthen [corticotropin-(1–24)-peptide, comprising the biologically active fragment of corticotropin] was obtained from CIBA–GEIGY. All other reagents and chemicals were of the highest purity commercially available.

Animals and their treatment

Male Wistar rats weighing 300–350 g at the time of being killed were used in all experiments. They were kept under conditions of controlled lighting (06:00–18:00 h light, 18:00–06:00 h dark) and maintained in rooms at constant temperature (22°C) for at least 2 weeks before the experiment. The rats were maintained on a regular commercial diet (Fidelity Feed, Harden, N.S.W., Australia) *ad libitum* and given free access to drinking water. In all experiments the rats were killed by decapitation. For the diurnal-rhythm experiment, three groups of two rats each were killed at 3 h intervals, starting at 06:00 h. In experiments involving corticotropin injection, two groups of three rats each were injected intraperitoneally with 4 units (30 μ g) of Synacthen each at 06:30 h, control rats were injected with the corresponding volume of saline (0.9% NaCl), and all animals were killed at 09:00 h.

Preparation of the microsomal fraction and the soluble fraction of rat adrenals

Adrenal microsomal fraction was prepared essentially as outlined by Balasubramaniam *et al.* (1977a). Microsomal pellets were frozen in liquid N₂ and stored at –75°C until they were resuspended in 0.1 M-potassium phosphate buffer, pH 7.5, containing 2 mM-dithiothreitol, to a protein concentration of approx. 2 mg/ml, immediately before assay.

The high-speed supernatant (S₁₀₄ fraction) obtained after sedimentation of the microsomal fraction was used for the assay of cholesterol esterase and for the determination of cytoplasmic non-esterified and esterified cholesterol.

Enzyme assays

The assays of microsomal hydroxymethylglutaryl-CoA reductase and acyl-CoA:cholesterol acyltransferase were performed as described previously (Balasubramaniam *et al.*, 1976, 1977b). Hydroxymethylglutaryl-CoA reductase activity is

expressed as pmol of [¹⁴C]mevalonate formed/min per mg of microsomal protein. Acyl-CoA:cholesterol acyltransferase activity is expressed as pmol of [¹⁴C]cholesteryl oleate formed/min per mg of microsomal protein.

Cholesterol esterase activity was assayed essentially as described by Goodman (1969) for the assay of the liver enzyme. In order to minimize the cholesteryl ester content of S₁₀₄ fraction the fluffy layer floating on the top of S₁₀₄ fraction was removed. In the standard assay a portion of the S₁₀₄ fraction was taken such that 60–80 μ g of S₁₀₄-fraction protein was used per incubation in a total volume of 200 μ l containing 0.1 M-potassium phosphate buffer, pH 7.0. The reaction was initiated by the addition of [¹⁴C]cholesteryl oleate (0.1 μ Ci; 58.4 mCi/mmol) dissolved in 5 μ l of acetone, and was allowed to proceed for 20 min at 37°C in a shaking water bath before termination with 4 ml of chloroform/methanol (2:1, v/v). Carrier cholesterol (0.5 mg) and cholesteryl oleate (50 μ g) were then added to each tube, and [¹⁴C]cholesterol and [¹⁴C]cholesteryl oleate were isolated by t.l.c. on silica gel with n-heptane/diethyl ether/acetic acid (85:20:2, by vol.) as the developing solvent. Cholesterol esterase activity was expressed as the percentage incorporation of [¹⁴C]cholesteryl oleate into [¹⁴C]cholesterol/min per mg of S₁₀₄-fraction protein. The amount of endogenous cholesteryl esters in each assay was only 5–10% (1 μ g) of the amount of exogenous ¹⁴C-labelled substrate (10 μ g) added to the incubation mixture. This minimized the effect of changes in the endogenous substrate on the activity of the enzyme.

Analytical determinations

The amounts of cholesterol and cholesteryl esters in the microsomal preparations and S₁₀₄ fractions were determined by g.l.c. after separation by t.l.c. by a previously described method (Brown *et al.*, 1975). [³H]Cholesteryl oleate, [¹⁴C]cholesterol and 5 α -cholestane were used as internal standards to correct for procedural losses. The tissue contents of cholesterol and cholesteryl oleate are expressed as μ g of each/mg of protein in the respective tissue fraction.

Corticosterone concentrations in rat plasma were determined by specific radioimmunoassay (Gomez-Sanchez *et al.*, 1975). Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard.

Statistical analysis

Results are expressed as means $\pm$ s.d. The probability (*P*) of a significant difference was assessed by Student's *t* test.

Results

Characterization of adrenal cholesterol esterase

To establish optimal conditions for the assay of cholesterol esterase activity rat adrenals were pooled and the S_{104} fraction was prepared and used under various assay conditions. Fig. 1 shows that the hydrolysis of the ester was linear for up to 20 min and with the amount of enzyme up to at least 120 μ g of protein. Saturation of the esterase activity occurred at a cholesteryl oleate substrate concentration of 5 μ M (apparent K_m 1.6 μ M). The pH optimum of the enzyme was 7.

Diurnal rhythm in the activity of adrenal cholesterol esterase and plasma corticosterone concentration

Rats exposed to repetitive dark and light cycles for 2 weeks were killed at 3 h intervals over a 24 h period and the various assays were performed. The activity of cytoplasmic cholesterol esterase in the adrenal, as shown in Fig. 2, exhibited diurnal variation with peak value at the onset of darkness ($13.5 \pm 2.9\%$); this was significantly higher ($P <$

0.01) than the minima observed at 03:00 and 15:00 h ($7.0 \pm 2.1\%$). A smaller peak in esterase activity appeared at the end of the dark period, and this was also found to have a significantly higher value than that observed at the minima at 03:00 and 15:00 h. In agreement with the previous observation (Gomez-Sanchez *et al.*, 1976), the plasma corticosterone concentration also exhibited diurnal rhythm with a broad peak at around 18:00 h (Fig. 2). The concentration of cytoplasmic esterified cholesterol, which was observed to build up during the periods of low esterase activity, diminished substantially during the periods of peak activity of the esterase. The non-esterified cholesterol concentration in the cytoplasm did not show marked variation over the 24 h period except for a slight but significant decrease 3 h after the onset of darkness.

Diurnal rhythm in the activities of microsomal acyl-CoA:cholesterol acyltransferase and hydroxymethylglutaryl-CoA reductase in the adrenal gland

The activity of acyl-CoA:cholesterol acyltransferase exhibited a characteristic rhythm showing a trough, with minimum value occurring 3 h after the

Fig. 1. Determination of optimal conditions for the assay of neutral cholesteryl esterase activity in rat adrenal gland. Samples of S_{104} fractions (containing 80 μ g of protein) and [14 C]cholesterol oleate (20 μ M) were incubated at 37°C in the standard incubation mixture in a final volume of 0.2 ml. In all except (a) the incubation time was 20 min. In (b) the amount of S_{104} protein per flask was varied; in (c) the pH was varied; in (d) the amount of [14 C]cholesteryl oleate per tube was varied. Experimental details are given in the Materials and methods section. All results are expressed as percentages of [14 C]cholesteryl oleate converted into cholesterol.

Fig. 2. Diurnal changes in the activity of cholesterol esterase and in the concentration of esterified and non-esterified cholesterol in the S_{104} fraction of the adrenal gland

Three groups of two rats each, adapted to repetitive dark and light cycles for 2 weeks, were killed every 3 h during the 24 h period. Blood was collected, microsomal fraction and S_{104} fraction from adrenal gland were prepared and various assays were made as indicated: (a) plasma corticosterone concentration; (b) cholesterol esterase activity; (c) cytoplasmic esterified (▲) and non-esterified (●) cholesterol concentrations. Experimental details are given in the Materials and methods section. Each value is a mean of values obtained from six animals. The bars represent standard deviations.

onset of darkness (Fig. 3). As demonstrated previously (Balasubramaniam *et al.*, 1977b), the activity of hydroxymethylglutaryl-CoA reductase also exhibited diurnal rhythm, with a peak of maximum value occurring 3 h after the onset of darkness. The microsomal concentration of non-esterified cholesterol showed a decrease of 50% of its basal value at the onset of darkness followed by a gradual increase to basal value 6 h later (Fig. 3).

Effect of acute corticotropin administration on the activities of cholesterol esterase, acyl-CoA:cholesterol acyltransferase and hydroxymethylglutaryl-CoA reductase

Intraperitoneal administration of corticotropin 2–3 h before the animals were killed resulted in the depletion of cytoplasmic cholesteryl esters and a 3-fold increase in the activity of cholesterol esterase in the adrenal gland (Table 1). This was accom-

Fig. 3. Diurnal rhythm in the activities of acyl-CoA:cholesterol acyltransferase and hydroxymethylglutaryl-CoA reductase and in concentration of microsomal non-esterified cholesterol in the adrenal gland

Microsomal fraction from the experiment described in Fig. 2 was used for various assays: (a) hydroxymethylglutaryl-CoA reductase activity; (b) cholesterol acyltransferase activity; (c) microsomal non-esterified cholesterol concentration. Experimental details are given in the Materials and methods section. Each value is a mean of values obtained from six animals.

panied by a 20-fold increase in the corticosterone concentration in the plasma. The activity of acyl-CoA:cholesterol acyltransferase, on the other hand, was decreased significantly to 30% of the control value, with a concomitant increase in hydroxymethylglutaryl-CoA reductase activity. Corticotropin administration also led to significant decrease in microsomal and cytoplasmic non-esterified cholesterol.

Discussion

The results presented here demonstrate that the two enzymes cholesterol esterase and acyl-

CoA:cholesterol acyltransferase in the adrenal gland are actively regulated during adrenal cholesterol metabolism. The results also indicate that the regulation of plasma corticosterone concentration is closely linked to the regulation of cholesterol esterase. These studies suggest that the characteristic rhythmic changes observed in the activities of the esterase and the esterifying enzyme, as well as that of hydroxymethylglutaryl-CoA reductase, are mediated by corticotropin.

The pattern of diurnal changes in the activity of cholesterol esterase and cytoplasmic esterified cholesterol concentration provides a classical example of a relationship between enzyme activity

Table 1. Effect of acute corticotropin treatment on the activities of cholesterol esterase, acyl-CoA:cholesterol acyltransferase and hydroxymethylglutaryl-CoA reductase and on the cellular cholesterol content in the adrenal gland

Groups of rats each consisting of three animals were given corticotropin in saline or saline only intraperitoneally at 06:30h and killed 2.5h later. The adrenals and plasma were collected and various assays made as described in the Materials and methods section.

Treatment	Activity of cholesterol esterase in S ₁₀₄ fraction (% of ¹⁴ C incorporated into cholesterol/min per mg of protein)	Cholesterol in S ₁₀₄ fraction (μg/mg of protein)		Enzyme activity (pmol/min per mg of protein)		
		Esterified	Non-esterified	Cholesterol acyltransferase	Hydroxymethylglutaryl-CoA reductase	Microsomal non-esterified cholesterol (μg/mg of protein)
Control	6.7 ± 3.1	6.3 ± 2.1	1.70 ± 0.16	145 ± 7	32 ± 5	51 ± 3
Control + corticotropin	19.8 ± 1.1	2.1 ± 0.4	1.22 ± 0.15	65 ± 7	72 ± 17	35 ± 5
P	<0.01	<0.01	<0.05	<0.01	<0.01	<0.05

and substrate concentration *in vivo* under normal physiological conditions (Fig. 2). The sharp rise in the activity of cholesterol esterase at the onset of darkness is accompanied by a sharp decline in the cytoplasmic concentration of esterified cholesterol. The accumulation of cytoplasmic esterified cholesterol, on the other hand, occurs when the activity of the enzyme falls. Thus, over a 24h period of equal light and dark cycles, the fluctuations in the adrenal cellular esterified cholesterol are closely related to the activity of cytoplasmic cholesterol esterase.

In the current studies the major diurnal peak in the activity of cholesterol esterase occurs when the plasma corticosterone concentration is at its diurnal maximum (Fig. 2). Pituitary corticotropin concentration has also been shown to exhibit a characteristic diurnal rhythm, with its peak at the onset of darkness (Ixart *et al.*, 1977). Acute administration of corticotropin increases both the esterase activity and plasma corticosterone concentration simultaneously (Table 1). Taken together, these observations suggest that the diurnal rhythm in the plasma corticosterone concentration is generated through diurnal changes in esterase activity caused by rhythmic fluctuations in plasma corticotropin concentration. However, the observation of a peak in the activity of the esterase at the end of the dark period, when there is neither an increase in pituitary corticotropin concentration nor an increase in plasma corticosterone concentration, suggests that non-esterified cholesterol released by hydrolysis of cytoplasmic esterified cholesterol cannot be utilized for corticosterone formation in the absence of corticotropin. On the other hand, it is possible that in the absence of corticotropin another enzyme in the corticosterone-biosynthetic pathway becomes rate-limiting.

An interesting observation in the present study is the characteristic diurnal rhythm exhibited by acyl-CoA:cholesterol acyltransferase. The profile of the rhythm is exactly the mirror image of the pattern of the diurnal rhythm exhibited by hydroxymethylglutaryl-CoA reductase (Fig. 3). The activity of the esterifying enzyme is minimum at a time when the activity of hydroxymethylglutaryl-CoA reductase is at its peak. Such a reciprocal relationship in the activities of these two enzymes is observed when rats are fed with a diet supplemented with cholesterol (Balasubramaniam *et al.*, 1978; Mitropoulos *et al.*, 1978). Moreover, it has been shown that acyl-CoA:cholesterol acyltransferase is regulated mainly by the supply of substrate for the enzyme (Nilsson, 1975). The diurnal fall in the activity of the enzyme may therefore be due to diurnal removal of its cholesterol substrate pool from the environment of the enzyme. In support of this suggestion is the observation that over the 24h period non-esterified cholesterol in the microsomal fraction was gradually

depleted, exhibiting a diurnal-rhythm pattern similar to that of the activity of the esterifying enzyme (Fig. 3). The diurnal decrease in microsomal non-esterified cholesterol at the onset of darkness strongly suggests that cholesterol of endoplasmic-reticular-membrane origin is also utilized for corticosterone formation.

Acute administration of corticotropin causes a decrease in microsomal non-esterified cholesterol with simultaneous decrease in the activity of acyl-CoA:cholesterol acyltransferase and enhancement in the activity of hydroxymethylglutaryl-CoA reductase, thus resembling the events occurring during the first 3 h after the onset of darkness in the diurnal cycle. This further confirms that corticotropin mediates the diurnal changes observed in the activities of acyl-CoA:cholesterol acyltransferase and hydroxymethylglutaryl-CoA reductase by altering the non-esterified cholesterol concentration in the environment of these enzymes.

The results obtained in the present investigation clearly demonstrate that there is a co-ordination in the regulation of the activities of these three enzymes during adrenal cholesterol metabolism. The sudden or diurnal release of corticotropin into the blood circulation activates the esterase through a cyclic AMP-dependent protein kinase (Trzeciak & Boyd, 1973; Pittman & Steinberg, 1977), accelerating the release of non-esterified cholesterol, which is rapidly converted into steroid hormones. The corticotropin also, by an unknown mechanism, shifts the non-esterified cholesterol from the cytoplasm and from the endoplasmic-reticular membrane, probably for corticosterone synthesis. This removal of non-esterified cholesterol, in turn, activates cholesterol synthesis through enhancement of hydroxymethylglutaryl-CoA reductase activity and decreases esterification by suppression of acyl-CoA:cholesterol acyltransferase activity.

The present studies also clearly suggest that corticotropin has two independent roles in adrenal

cholesterol metabolism: one to stimulate cholesterol esterase activity and the other to enhance the transfer of non-esterified cholesterol from the cytoplasm and endoplasmic-reticular membrane into the mitochondria for further metabolism.

We thank Mrs. Mary Rowe, Mr. Jan Ruys and Mrs. Alison Reeves for their skilful assistance and Dr. L. A. Simons for his helpful suggestions.

References

- Balasubramaniam, S., Goldstein, J. L., Faust, J. R. & Brown, M. S. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 2564–2568
- Balasubramaniam, S., Goldstein, J. L., Faust, J. R., Brunschede, G. Y. & Brown, M. S. (1977a) *J. Biol. Chem.* **252**, 1771–1779
- Balasubramaniam, S., Goldstein, J. L. & Brown, M. S. (1977b) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 1421–1425
- Balasubramaniam, S., Mitropoulos, K. A. & Venkatesan, S. (1978) *Eur. J. Biochem.* **90**, 377–383
- Boyd, G. S. & Trzeciak, W. H. (1973) *Ann. N.Y. Acad. Sci.* **212**, 361–371
- Brown, M. S., Faust, J. R. & Goldstein, J. L. (1975) *J. Clin. Invest.* **55**, 783–793
- Gomez-Sanchez, C., Holland, O. B., Higgins, J. R., Kem, D. C. & Kaplan, N. M. (1975) *Endocrinology* **99**, 567–572
- Goodman, D. S. (1969) *Methods Enzymol.* **15**, 522–537
- Ixart, G., Szafarczyk, A., Belugou, J. L. & Assenmacher, I. (1977) *J. Endocrinol.* **72**, 113–120
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Mitropoulos, K. A., Balasubramaniam, S., Venkatesan, S. & Reeves, B. E. A. (1978) *Biochim. Biophys. Acta* **530**, 99–111
- Nilsson, A. (1975) *Eur. J. Biochem.* **51**, 337–352
- Pittman, R. C. & Steinberg, D. (1977) *Biochim. Biophys. Acta* **487**, 431–440
- Trzeciak, W. H. & Boyd, G. S. (1973) *Eur. J. Biochem.* **37**, 327–333