Circadian rhythm in hepatic low-density-lipoprotein (LDL)-receptor expression and plasma LDL levels

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On the basis of studies *in vivo* and *in vitro* that involved the use of pharmacological amounts of drugs and hormones or excess cholesterol supplementation, the expression of the low-density lipoprotein (LDL) receptor appears to be tightly coupled to the regulation of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase activity and to extracellular levels of LDL. The present study was undertaken to see how these three entities are regulated under normal physiological conditions over a 24 h period. The results show that, in the rat, hepatic LDL-receptor expression and plasma LDL levels exhibit diurnal periodicity, with a 2–3-fold difference between the peak and trough of each rhythm. Both rhythms showed high inverse correlation ($r = -0.86$, $P < 0.01$), plasma LDL levels being lowest at the onset of darkness when LDL-receptor expression was at its peak. The results also showed that the LDL-receptor protein in rat liver has a shorter half-life than that reported for cultured fibroblasts or HepG2 cells. The maximal expression of the LDL receptor occurred several hours before the peak activity of HMG-CoA reductase and appeared not to be influenced by cellular or membrane cholesterol levels during the 24 h cycle. Treatment with dexamethasone increased the LDL-receptor activity significantly at both the lowest and highest points of the rhythm, but the receptor rhythm was still maintained, suggesting that the signal for the circadian variation of the receptor expression is not mediated by adrenal hormones.

INTRODUCTION

By regulating the expression of the low-density lipoprotein (LDL) receptor and the activity of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, cells *in vivo* can obtain cholesterol either from plasma lipoproteins or from cholesterol synthesis *de novo* (Goldstein and Brown, 1984). Within the cells both the receptor, most of which occurs in the liver, and the reductase are subject to feedback regulation by cholesterol through repression or de-repression of the sterol regulatory elements of the genes encoding these two proteins. Plasma LDL levels, in part, are dictated by the balance between the rates of synthesis of the receptor and the reductase. Under a variety of experimental conditions the modulation of the expression of both of these proteins appears to be tightly co-ordinated.

Most of the information about the regulation of the LDL receptor, plasma LDL levels and HMG-CoA reductase has been derived from perturbation studies involving extremes of drug, dietary or hormonal manipulation. This does not provide a true picture of the sequence of the metabolic events involved in the regulatory process. How these three entities are regulated under normal physiological conditions is not known. It was therefore the aim of the present study to examine how the LDL receptor activity and plasma LDL levels vary over a 24 h period in the absence of any experimental manipulations and to relate any observed variations to the well-defined diurnal rhythm of HMG-CoA reductase (Edwards et al., 1972). To achieve this we carried out our studies in normal rats, using a sensitive method to measure hepatic LDL-receptor protein. The results clearly indicate that hepatic LDL-receptor expression and plasma LDL levels exhibit diurnal periodicity. Both show a strong inverse correlation, plasma LDL levels being the lowest at the time when the LDL-receptor expression is maximal. The reductase and the receptor rhythms appear not to be influenced by cellular or membrane non-esterified cholesterol, which remained constant throughout the 24 h cycle. Dexamethasone, however, enhanced the amplitude of the receptor rhythm, but did not abolish it, indicating that the signal for the rhythm is not mediated by adrenal hormones.

MATERIALS AND METHODS

Animals

Male Wistar rats weighing 200–250 g were acclimatized to 12 h-light/12 h-darkness cycles (lights on at 06:00 h) and maintained in rooms at constant temperature (22 °C) for at least 2 weeks before use. Throughout these periods of adaptation rats had full access to water and rat chow (Norco Co-operative, Lismore, N.S.W., Australia), which contained (by wt.) protein 21 %, carbohydrate 63 %, fat 4 %, crude fibre 3 %, moisture 6 %, fatty acid 0.8 %, vitamins and minerals including antioxidants 0.5 %, and cholesterol 0.01 %. Groups of six rats each were killed at 3 h intervals over a 24 h period. Whole liver and blood samples were collected at every time point. Rats were also treated with dexamethasone phosphate (1.6 mg/kg body wt.; 0.1 ml subcutaneous injections) daily for 7 days; control rats were injected with 0.1 ml of 0.9 % NaCl only. Rabbits were fed on a diet supplemented with cholesterol (1 %, w/w) for 5 days before harvesting the plasma LDL.

LDL preparation

Rat and rabbit LDL was isolated in the density range 1.019–1.055 g/ml by preparative ultracentrifugation.

Abbreviations used: LDL, low-density lipoprotein; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; ECL, enhanced chemiluminescence.

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Preparation of liver membrane fractions and solubilization

For the LDL-receptor assay, a crude ultracentrifugal fraction (8000–100000 *g*) was prepared from liver as previously described (Kovanen et al., 1979). A solution containing 50 mM Hepes (pH 7.4), 100 mM NaCl, 1 mM phenylmethanesulphonyl fluoride, 10 mM *N*-ethylmaleimide, 10 mM EDTA and 10 mM EGTA was used as the homogenizing buffer. The pelleted membranes were suspended in 50 mM Hepes/100 mM NaCl and solubilized by adding an equal volume of 2% (w/v) SDS/10% (v/v) glycerol/0.005% (w/v) Bromophenol Blue in the same buffer. For the assay of the activity of HMG-CoA reductase, the microsomal membrane fractions were prepared as above, but in 0.25 M sucrose (pH 7.4)/2 mM EDTA. The membranes were suspended in 0.25 M sucrose (pH 7.4)/3 mM imidazole. Protein was determined by the method of Lowry et al. (1951).

Quantification of the LDL receptor

Solubilized membrane fractions (20 μg of protein/lane) were subjected to electrophoresis on 3–12% linear gradient SDS/polyacrylamide gels at 175 V for 3 h along with Rainbow molecular-mass markers (Pharmacia LKB, Uppsala, Sweden). The separated proteins were electrotransferred from the gels on to nitrocellulose membranes at 30 V for 16 h. Incubation with ligands was performed by the method of Daniel et al. (1982), with the following modifications. The nitrocellulose blots were blocked in buffer A (50 mM Tris/HCl, 2 mM $CaCl_2$, 90 mM NaCl at pH 8, containing 10% skim-milk powder). Rat or rabbit LDL was biotinylated with D-biotin *N*-hydroxysuccinimide ester as described by Roach and Noel (1987), and this was used in the ligand-binding assay [7.5 μg of protein (biotinylated rat or rabbit LDL)/ml of buffer A]. After washing in buffer B (containing 1% skim-milk powder), LDL receptors were detected by incubating with 4 μl of streptavidin–horseradish peroxidase complex/ml of buffer B and by performing the enhanced chemiluminescence (ECL) procedure (Amersham International, Amersham, Bucks, U.K.). Briefly, blots were immersed in a solution containing luminol and exposed for a few minutes to Kodak XAR-5 X-ray film. The chemiluminescence reaction produced clear and intense bands. The bands on the X-ray film corresponding to the receptors were then quantified with a scanning laser densitometer (LKB Ultrascan). In addition to using the biotinylated-LDL ligand-binding procedure for quantifying LDL-receptor activity, monospecific polyclonal anti-LDL-receptor antibodies coupled to the ECL procedure were also used to quantify the LDL-receptor mass. Monospecific polyclonal anti-LDL-receptor antibodies were prepared by injecting purified bovine adrenal-cortex LDL receptors into New Zealand White rabbits (Beisiegel et al., 1981).

Assay of HMG-CoA reductase

The rate of conversion of [^{14}C]HMG-CoA into [^{14}C]mevalonate in microsomes was measured by t.l.c. as previously described (Balasubramaniam et al., 1976).

Other analytical determinations

The amount of non-esterified cholesterol in whole liver homogenates or in microsomal membranes was determined by g.l.c. (Brown et al., 1975). Cholesterol in plasma and in LDL fractions was determined by automated enzymic methods (Boehringer-Mannheim, Mannheim, Germany).

RESULTS

To detect and quantify LDL-receptor protein in rat liver, crude hepatic microsomal membranes were first solubilized and proteins were separated by gradient gel electrophoresis. The proteins were transblotted on to nitrocellulose membranes and ligand-blotted with biotinylated rat or rabbit LDL. The bound LDL was then detected by the ECL procedure. This provided a very sensitive method for measuring LDL receptors. For a normal rat, a single band was observed for the LDL receptor by using rat LDL as the ligand, and this corresponded to a 130 kDa protein, as the electrophoresis was performed under non-reducing conditions (Figure 1a). Rabbit LDL under the same conditions also bound to the rat LDL receptor with similar affinity (Figure 1b).

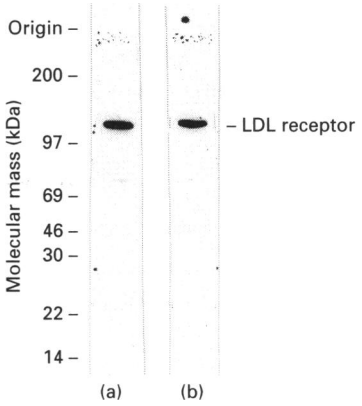

Figure 1 Detection of the LDL receptor in rat liver by ligand blotting with (a) rat LDL and (b) rabbit LDL

Microsomal membranes prepared from rat liver cells were solubilized in SDS and subjected to SDS/PAGE (3–12% linear gradient, under non-reducing conditions). The nitrocellulose blots were incubated with (**a**) biotinylated rat LDL or (**b**) biotinylated rabbit LDL and detected by using the ECL system as described in the text. Rainbow molecular-mass standards (myosin, 200 kDa; phosphorylase b, 97 kDa; BSA, 69 kDa; ovalbumin, 46 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 22 kDa; lysozyme, 14 kDa) were run on adjacent lanes.

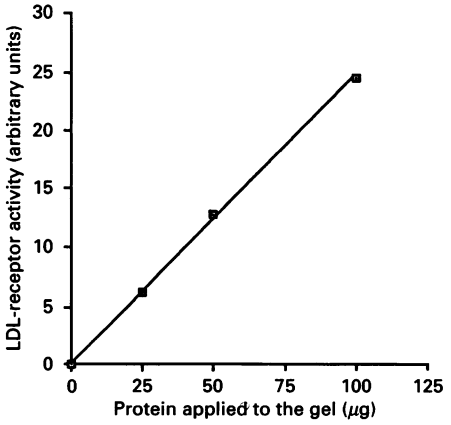

Figure 2 Quantification of LDL receptor by densitometric scanning after ligand binding with rabbit LDL

Crude membranes prepared from rat liver were solubilized, and increasing amounts of protein (0–100 μg) were subjected to SDS/PAGE (3–12% linear gradient, under non-reducing conditions). The nitrocellulose blots were incubated with biotinylated rabbit LDL, and the LDL-receptor bands were quantified by using the ECL system as described in the text.

Table 1 Feeding patterns and food intake during the diurnal cycle

Food intake was monitored every 6 h. Results are expressed as mean ± S.E.M. for six rats.

	Light phase		Dark phase	
Period	06:00–12:00 h	12:00–18:00 h	18:00–24:00 h	24:00–06:00 h
Chow consumed (g/250 g rat)	0.5 ± 0.2	0.9 ± 0.4	13.8 ± 2.9	16.7 ± 3.5

Hence, in the subsequent studies rabbit LDL was used because of the ease with which it could be obtained. Monospecific polyclonal anti-LDL-receptor antibodies, also coupled to the ECL procedure, were used to confirm the identity of the LDL-receptor bands. The intensities of the bands obtained by ligand binding and that obtained by immunoblotting were proportional to the amount of protein applied to the gel. Figure 2 shows that the LDL-receptor receptor activity, obtained by ligand binding with rabbit LDL, increases linearly with increasing amounts of crude membrane protein (0–100 μg) applied to the gel. The linearity of response was maintained up to 100 μg of protein loaded.

Table 1 shows the feeding pattern of the rats over the 24 h period. Most of the daily food intake occurred during the dark phase at a constant rate (2.5 g/h). Negligible amounts of food were consumed during the light phase (0.11 g/h).

As shown in Figure 3(b), the LDL-receptor activity exhibited diurnal rhythm with a peak value at the onset of darkness which was significantly higher than the minimum value observed at 09:00 h during the light period (Table 2). Figure 4 shows the intensity of LDL-receptor bands observed at the diurnal maximum and minimum as determined by ligand binding. The immunoblotting procedure showed that the peak activity of the LDL receptor (235 ± 19 arbitrary units; mean ± S.E.M, $n = 6$) observed at the onset of darkness was significantly higher ($P < 0.001$) than the minimum value (100 ± 12, $n = 6$) observed at 09:00 h during the light period.

The plasma LDL concentration exhibited diurnal rhythm

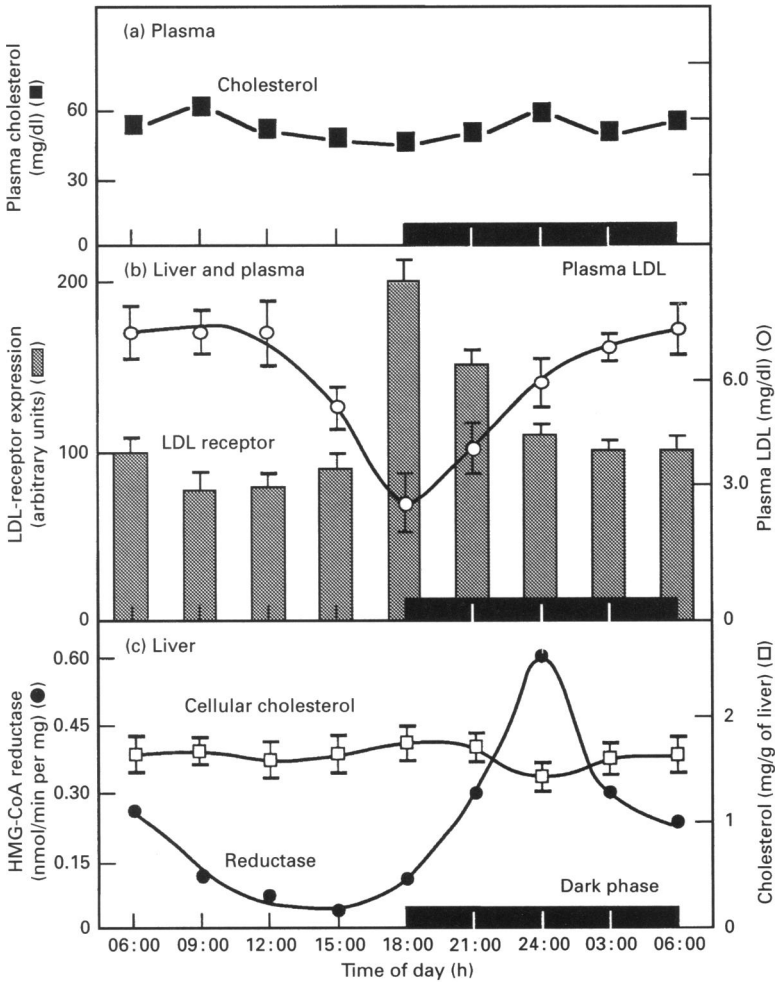

Figure 3 Diurnal changes in the activities of the hepatic LDL receptor and HMG-CoA reductase and in the cholesterol concentration of plasma, LDL and whole liver homogenates

Groups of six 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 and microsomal membrane fractions were prepared from the livers. The various assays were then performed as described in the text. (**a**) Plasma cholesterol concentration, (**b**) LDL-receptor activity and plasma LDL levels. (**c**) HMG-CoA reductase activity and cellular cholesterol concentration.

Table 2 Significance of the differences between the diurnal maximum and minimum of the activities of the LDL receptor and HMG-CoA reductase, plasma LDL concentration and cellular and membrane non-esterified cholesterol

Results are expressed as means ± S.E.M. for six rats used at each time point. Statistical analysis of differences was done by Student's *t* test: N.S., not significant.

	At minimum	At maximum	Probability of significance
LDL receptor expression (arbitrary units)	82 ± 9	204 ± 16	$P < 0.001$
Plasma LDL (mg of cholesterol/ 100 ml)	2.81 ± 0.65	6.82 ± 0.82	$P < 0.001$
HMG-CoA reductase activity (pmol/min per mg of protein)	96 ± 7	650 ± 62	$P < 0.001$
Cellular non-esterified cholesterol (mg/g of liver)	1.50 ± 0.21	1.80 ± 0.32	N.S.
Membrane non-esterified cholesterol (nmol/mg of protein)	60.5 ± 1.3	65.8 ± 2.9	N.S.

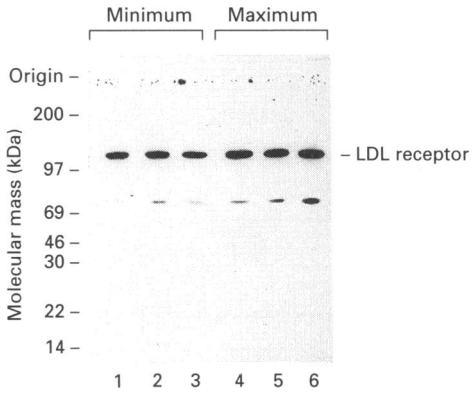

Figure 4 LDL-receptor expression in rat liver at the two time points when the diurnal maximum and minimum occur

LDL-receptor protein was separated from solubilized liver membranes, electrotransferred to nitrocellulose blots, incubated with biotinylated rabbit LDL and quantified as described in the text. Receptor activities from three rats killed at 09:00 (lanes 1–3) and at 18:00 h (lanes 4–6) are shown.

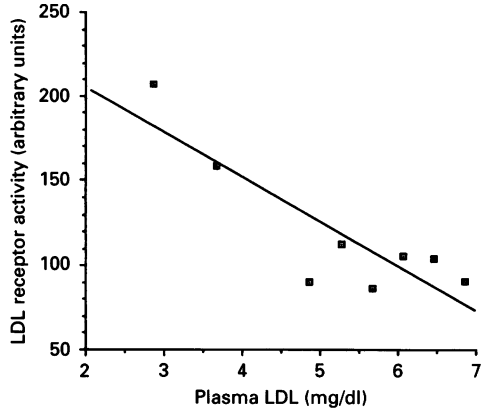

Figure 5 Correlation between rat hepatic LDL-receptor activity and plasma LDL cholesterol concentration over the 24 h cycle

Values for the LDL-receptor activity and plasma LDL cholesterol were obtained from Figure 3.

(Figure 3b) with the lowest value at the onset of darkness, which was significantly lower than the value obtained at 12:00 h during the light period (Table 2). The LDL-receptor activity in liver membranes was compared with the values for the concentration of LDL in rat plasma over the 24 h cycle. There was a significant inverse correlation ($r = -0.86$; $P < 0.01$) between LDL-receptor activity and plasma LDL cholesterol concentration, as shown in Figure 5.

HMG-CoA reductase activity (Figure 3c) exhibited a maximum at the mid-dark phase 6 h after the peak activity of the LDL receptor. The plasma cholesterol concentration showed no significant changes during the 24 h period (Figure 3a). Non-esterified cholesterol, in the whole liver homogenates and in microsomal membranes, was not significantly altered during the 24 h cycle (Figure 3c, Table 2). Administration of dexamethasone for 7 days increased the LDL receptor activity significantly both at the diurnal minimum (control and treated, 82 ± 9 and 213 ± 15 arbitrary units respectively; $n = 6$; $P < 0.001$) and at the diurnal maximum (control and treated, 204 ± 16 to 395 ± 21; $n = 6$; $P < 0.001$).

DISCUSSION

The present study clearly demonstrates for the first time that hepatic LDL-receptor expression and plasma LDL levels show characteristic circadian rhythm, displaying high amplitudes, and that both are actively regulated under normal physiological conditions. The strong inverse correlation between receptor expression and LDL levels, observed over the 24 h period, is consistent with a cause–effect relationship between the two (Figure 5). Our results also show that the maximal expression of the LDL receptor occurred several hours before the peak activity of HMG-CoA reductase, suggesting that the receptor rhythm is independent of the rhythmic changes in HMG-CoA reductase. Both the LDL receptor and HMG-CoA reductase rhythms appear not to be influenced by cellular or membrane non-esterified cholesterol. The rhythms observed in the present study do not seem to be influenced by the feeding pattern of the animals. Our results also show that adrenal hormones are not directly involved in the generation of the receptor rhythm, although they can influence the rhythm.

We were able to demonstrate the existence of diurnal rhythm in hepatic LDL-receptor expression, which others had failed to observe (Erickson et al., 1989), partly because the method used in our study is much more sensitive than other methods used previously to detect and quantify the LDL-receptor activity, and partly because we measured the receptor levels at regular intervals over a 24 h period. The combination of commercially available detection reagents coupled with minor modifications to existing techniques (Roach and Noel, 1987; Wade et al., 1985) resulted in this increased sensitivity.

The results in the present study demonstrate that LDL-receptor ligand-binding activity represents LDL-receptor protein mass. This correlation between mass and binding activity has also been previously demonstrated (Soutar et al., 1986). The LDL-receptor diurnal rhythm observed in the present study is therefore the result of diurnal changes in protein expression. Whether the diurnal variations observed in the receptor expression are a

direct result of alterations in receptor mRNA level is yet to be established. However, it is now known that any alteration in LDL-receptor expression is closely matched by a parallel alteration in LDL-receptor mRNA level (Soutar and Knight, 1990). Thus it is likely that the diurnal rhythm in the LDL-receptor expression observed in our study is controlled at the level of gene transcription, although post-transcriptional levels of control cannot be ruled out. It is noteworthy that the diurnal rhythm of HMG-CoA reductase expression is also closely paralleled by the level of its mRNA (Clarke et al., 1984).

An interesting observation in the present study is that the LDL-receptor protein in the rat turns over rapidly. The peak LDL-receptor expression, at the onset of darkness, is decreased by half within 6 h, indicating that the LDL-receptor protein in rat hepatocytes has a shorter half-life than that of 9–14 h observed for the receptor in HepG2 cells or cultured human fibroblasts (Knight et al., 1987; Tam et al., 1991). The rapid turnover of the rat LDL-receptor protein may in part be due to its larger size compared with other species (Lee et al., 1989; Bishop, 1992). Our studies also showed that LDL receptor activity doubles within 3–6 h. Taken together, these observations indicate that the LDL-receptor expression in the rat can be suppressed or induced rapidly under normal physiological conditions that do not involve any drug, dietary or hormonal manipulation.

One mechanism by which the LDL receptor expression can be modulated is by sterols (Soutar and Knight, 1990). In the present study, despite no significant alterations in cellular or membrane non-esterified cholesterol levels during the 24 h cycle, there was a 2-fold variation in the receptor expression during this period. This implies that the diurnal rhythm of the LDL receptor is not regulated by non-esterified cholesterol. Another mechanism by which the receptor can be modulated is by induction or repression by hormones (Szanto et al., 1992), which is independent of sterols. The diurnal peak of plasma corticosterone is known to occur at the onset of darkness (Beins et al., 1982). Since the diurnal peak of the LDL-receptor expression in our study also occurs at the onset of darkness, and since dexamethasone has been shown to regulate the LDL-receptor activity (Salter et al., 1987), this raised the possibility that adrenal hormones play some role in the generation of the receptor rhythm. In the present study, however, treatment with dexamethasone, a synthetic hormone known to suppress the secretion of corticotropin ('ACTH') and thus the release of adrenal hormones, did not abolish the LDL-receptor rhythm, but resulted in a several-fold increase in the receptor expression at both the peak and the nadir of the rhythm. This suggests that the signal for the receptor rhythm is not mediated through diurnal changes in the release of adrenal hormones.

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REFERENCES

Balasubramaniam, S., Goldstein, J. L., Faust, J. R. and Brown, M. S. (1976) Proc. Natl. Acad. Sci. U.S.A. **73**, 2564–2568
Beins, D. M., Vining, R. and Balasubramaniam, S. (1982) Biochem. J. **202**, 631–637
Beisiegel, U., Kita, T., Anderson, R. G. W., Schneider, W. J., Brown, M. S. and Goldstein, J. L. (1981) J. Biol. Chem. **256**, 4071–4078
Bishop, R. W. (1992) J. Lipid Res. **33**, 549–557
Brown, M. S., Dana, S. E. and Goldstein, J. L. (1975) J. Biol. Chem. **250**, 4025–4027
Clarke, F. C., Fogelman, A. M. and Edwards, P. A. (1984) J. Biol. Chem. **259**, 10439–10447
Daniel, T. O., Schneider, W. J., Goldstein, J. L. and Brown, M. S. (1982) J. Biol. Chem. **256**, 4606–4611
Edwards, P. A., Muroya, H. and Gould, R. G. (1972) J. Lipid Res. **13**, 396–401
Erickson, S. K., Jaeckle, S., Lear, S. R., Brady, S. M. and Havel, R. J. (1989) J. Lipid Res. **30**, 1763–1771
Goldstein, J. L. and Brown, M. S. (1984) J. Lipid Res. **25**, 1450–1461
Knight, B. L., Patel, D. D. and Soutar, A. K. (1987) Eur. J. Biochem. **163**, 189–196
Kovanen, P. T., Brown, M. S. and Goldstein, J. L. (1979) J. Biol. Chem. **254**, 11367–11373
Lee, L. Y., Mohler, W. A., Schafer, B. L., Freudenberger, J. S., Byrne-Connolly, N., Eager, K. B., Mosley, S. T., Leighton, J. K., Thrift, R. N., Davis, R. A. and Tanaka, R. D. (1989) Nucleic Acids Res. **17**, 1259–1260
Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
Roach, P. D. and Noel, S. (1987) J. Lipid Res. **28**, 1508–1514
Salter, A. M., Fisher, S. C. and Brindley, D. N. (1987) FEBS Lett. **220**, 159–162
Soutar, A. K., Harders-Spengel, K., Wade, D. P. and Knight, B. L. (1986) J. Biol. Chem. **261**, 17127–17133
Soutar, A. K. and Knight, B. L. (1990) Br. Med. Bull. **46**, 891–916
Szanto, A., Balasubramaniam, S., Roach, P. D. and Nestel, P. J. (1992) Biochem. J. **288**, 791–794
Tam, S. P., Brissette, L., Ramharack, R. and Deeley, R. G. (1991) J. Biol. Chem. **266**, 16764–16773
Wade, D. P., Knight, B. L. and Soutar, A. K. (1985) Biochem. J. **229**, 785–790

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