CMLS, Cell. Mol. Life Sci. 58 (2001) 1327–1332
1420-682X/01/091327-06 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Research Article

Production of butyrylcholinesterase by Caco-2 cells: lack of relationship with triglyceride production

E. W. Randell[a,b,*], B. Rogers[b] and E. Smith[b]

[a] Department of Laboratory Medicine, Faculty of Medicine, Health Sciences Centre, 300 Prince Philip Dr., St. John's, Newfoundland AIB 3V6 (Canada), Fax +1 709 777 6395, e-mail: erandell@morgan.usc.mun.ca
[b] Department of Biochemistry, Faculty of Science, Memorial University of Newfoundland, Science Building, Elizabeth Ave., St. John's, Newfoundland A1C 5S7 (Canada)

Received 23 April 2001; received after revision 25 May 2001; accepted 20 June 2001

Abstract. Elevated levels of butyrylcholinesterase activity occur under a number of hypertriglyceridemic conditions, including diabetes and obesity. This study examines whether butyrylcholinesterase activity has a direct effect on triglyceride production, using Caco-2 cells, a human intestinal adenocarcinoma cell line. Caco-2 cells were incubated with 500 µM oleate to stimulate triglyceride production, and butyrylcholinesterase activity was measured in the cellular homogenate. Butyrylcholinesterase activity was approximately 3×10^{-3} µmol/min per milligram protein. Although triglyceride production increased by almost five-fold after 18 h of stimulation with oleate, butyrylcholinesterase activity was not increased. Furthermore, inhibition of butyrylcholinesterase activity using 1 mM tetraisopropylpyrophosphoramide did not significantly affect triglyceride production or secretion. Human insulin (100 µU/ml) increased the production of butyrylcholinesterase without increasing triglyceride production. This demonstrates that stimulation of fatty acid production and butyrylcholinesterase activity occur by independent mechanisms and suggests that their correlation in hyperlipidemic conditions is not due to a direct relationship in production in situ.

Key words. Butyrylcholinesterase; cholinesterase; obesity; lipid; triglyceride; insulin; Caco-2; diabetes.

Butyrylcholinesterase (BuChE; E.C. 3.1.1.8), also known as plasma cholinesterase or pseudocholinesterase, is distinguished from acetylcholinesterase (E.C. 3.1.1.7) by being selectively and irreversibly inhibited by tetraisopropylpyrophosphoramide (iso-OMPA). While acetylcholinesterase is important for the control of acetylcholine-mediated cholinergic function, the function of BuChE has been the subject of controversy and to date its precise physiological function remains unknown. Apart from plasma, this enzyme is found in smooth muscle cells, liver, adipose tissue, intestinal villi, kidney tubules, and macrophages [1]. Plasma BuChE is secreted mainly from the liver.

A number of studies have documented a relationship between plasma BuChE and very low density lipoprotein (VLDL) levels in obese, diabetic, or certain other hypertriglyceridemic patients. Plasma BuChE is elevated in the majority of patients with high levels of VLDL [2–6]. Obese [3, 4] and diabetic patients [5, 6] with hypertriglyceridemia also have high levels of BuChE activity. There is also a parallel decrease in BuChE and triglyceride (TG) on treatment by diet or drugs. Levels of BuChE are highly correlated with TG levels in diabetic and obese children but not in non-diabetic or non-obese children [7]. Similar observations have been made in an-

* Corresponding author.

imals. Genetically obese (ob/ob) mice, diabetic (db/db) mice [5, 8], and Zucker fat rats [9] all show increased levels of plasma and liver BuChE activity and have altered VLDL metabolism. Furthermore, streptozotocin-induced diabetes in rats result in a concomitant increase in BuChE, TG, VLDL, and glycerol levels [10]. Control of the diabetes in these animals with insulin results in normalization of glucose, BuChE, TG, VLDL, and glycerol levels. These results suggest an intimate relationship between TG and VLDL metabolism and BuChE levels. Nevertheless, the precise stimuli for plasma BuChE activity remain unknown.

The purpose of this study was to investigate the relationship between BuChE activity and TG production at the cellular level in a cell line producing both TG and BuChE. This study documents the presence of BuChE activity in Caco-2 cells, a human intestinal adenocarcinoma cell line, and examines its relationship with TG production under hormonal stimulation with insulin and with stimulation by fatty acids.

Materials and methods

Materials

Chemicals used for this study were of the highest grade commercially available. Dibucaine, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), butyrylthiocholine iodide, hexane, diethyl ether, glacial acetic acid, oleic acid, bovine serum albumin (BSA, fatty acid free), iso-OMPA, and N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid] (HEPES), were all purchased from Sigma (St. Louis, Mo.) [2-^{3}H]glycerol (37 GBq/mmol) was purchased from Amersham Life Sciences (Arlington Heights, Il). Silica Gel G thin layer chromatography plates were purchases from Alltech Associates Inc. (Deerfield, Il, USA). Human insulin (Humulin) was purchased from Eli Lilly Canada (Scarborough, Canada). Caco-2 cells, an intestinal carcinoma cell line, were purchased from the American Type Culture Collection (Rockville, Md.). Tissue culture medium and supplies were purchased from Gibco-BRL (Burlington, Canada).

Cell culture

Caco-2 cells were initially grown in α-minimum essential media (MEM) containing 4.5 g/l glucose and 1% non-essential amino acids, supplemented with 20% fetal calf serum, 4 mM glutamine, 50 µg/ml penicillin, 50 µg/ml streptomycin, and 0.1% (v/v) fungizone. The cells were grown in six-well plates (12.5 cm^2/well) at 37 °C in a humidified atmosphere of 95% oxygen, 5% carbon dioxide. Cell medium was changed every 2–3 days.

Subculturing was performed by first washing the cells with Hank's balanced salt solution and then adding 0.25% trypsin-EDTA solution. After a 2 to 3-min incubation at room temperature, the trypsinized cell suspension was split at a ratio of 1:4 and fresh medium was added to each plate. Confluence was reached in an average of 14–20 days for each plate. All cells were studied between the 20–25th passage.

Oleic acid-albumin preparation

The oleic acid-albumin solution was prepared using the method described by Ellsworth et al. [11]. Briefly, 83 µmol of BSA was dissolved to 25 ml in M199 tissue culture medium containing 10 mM HEPES buffer solution, pH 7.4. The solution was heated gently to allow the BSA to dissolve. Oleic acid (250 µmol) was dissolved in 25 ml of M199 medium by gently heating. The two solutions were then mixed and the resulting oleic acid:BSA (3:1 mol:mol) solution was covered and stirred at room temperature overnight. An 83 µmol BSA sample was carried through the same procedure and finally diluted to 50 ml with M199 media but without oleic acid. This solution was used to treat cells in control experiments. Before the solutions were incubated with the Caco-2 cells they were filter sterilized using a 0.45 µm filter.

BuChE assay

Measurement of BuChE activity was carried out using a modification of the method described by Dietz et al. [12] using butyrylthiocholine iodide as a substrate. Briefly, assays were carried out in 0.423 mM DTNB-phosphate buffer solution, pH 7.4, to which cell sonicates (200 µl) were added with 7.5 mM butyrylthiocholine iodide in a final volume of 600 µl incubated at 37 °C. A blank consisting of all reagents plus 200 µl of phosphate buffered saline instead of cell sonicates was used to estimate butyrylthiocholine autolysis, which was subtracted from all experimental results. Spectrophotometric readings were taken at 410 nm at time 0 and 120 minutes after the preincubation phase (60 min). Enzyme activity was expressed as µmol/min per milligram of cellular protein. Protein concentration was measured by the method of Lowry et al. [13].

Stimulation of TG synthesis in Caco-2 cells

Confluent Caco-2 cells were washed twice in Hanks balanced salt solution and incubated with serum-free MEM medium for 24 h prior to any experimentation. After 24 h the serum-free medium was removed and the cells were washed three times with M199. Cells were then incubated with 2 ml of M199 buffered with 10 mM HEPES pH 7.4 at 37 °C containing 500 µmol/l oleate in a BSA-containing mixture and [^{3}H]-glycerol (4.5 µM) under standard tissue culture conditions. At selected time intervals cell medium was removed and placed on ice. The cell monolayer was rinsed twice with M199, twice with phosphate buffered saline and then scraped into 2 ml of phosphate buffered saline on ice. The removed medium was cen-

trifuged at 2000 g for 10 min to remove cellular debris. The 2 ml cell suspension was sonicated at setting 50 in 3 consecutive 10 sec pulses in a sonicator (Sonic Dismembrator; Quigley-Rochester, Rochester, N.Y.) to achieve a cell homogenate mixture. A 0.5-ml portion of the cell homogenate was removed for lipid extraction while the rest was stored frozen at −20 °C to be used to carry out BuChE assays and to measure protein concentration [13]. Cell media was also stored frozen at −20 °C for further experimentation.

Lipid extraction and purification

Lipid extraction was carried out according to the method of Folch et al. [14]. The triglycerides in the lipid extract were separated using Silica G TLC plates. The plate was then developed in a solvent system of hexane/diethyl ether/acetic acid (35/15/1). Once developed, the plate was allowed to dry and then stained using 2% phosphomolybdic acid in a 95% ethanol spray followed by heating at 150 °C for 5 min. The TG spots were recognized by pure standards and scraped into scintillation vials, and 10 ml of aqueous scintillation cocktail (Sigma) was added. The radioactivity in the TG spots was measured using a liquid scintillation counter.

Statistical analysis

Student's t-test for unpaired groups was used to compare data. Statistical significance was defined as $p < 0.05$.

Results

Stimulation of Caco-2 cells with an oleate-BSA (500 µM/l) complex resulted in increased TG production and secretion compared with unstimulated cells treated with fatty-acid free BSA. Figure 1 shows the time-dependent increase in cellular TG content measured after 3, 6, and 18 h of incubation. After 18 h the TG content was increased to 0.61×10^6 cpm/mg protein in oleate-stimulated cells compared with 0.12×10^6 cpm/mg protein in unstimulated cells. Less than 10% of TG was secreted into the cell-free medium. After 18 h of incubation TG secretion from unstimulated cells was 3.5×10^3 cpm/mg cellular protein and 3.27×10^4 cpm/mg cellular protein in oleate-stimulated cells.

No previous study has documented the production of BuChE activity by Caco-2 cells. The presence of BuChE activity in the cellular homogenate of Caco-2 cells at 0 and 18 h of incubation is shown in table 1. There was no significant difference in BuChE activity between unstimulated and oleate-stimulated cells. Furthermore, there was no evidence of BuChE activity secretion into the medium.

To determine whether BuChE activity is necessary for production of TG in Caco-2 cells, cells were stimulated with oleate in the presence or absence of iso-OMPA (fig. 2). There was marked inhibition of BuChE activity in the oleate-stimulated cells when they were incubated in the presence of 1 mM iso-OMPA. After a 6 h incubation period, no difference was observed in TG production between cells treated with or without 1 mM iso-OMPA.

Other metabolic and dietary factors may stimulate BuChE activity. Figure 3 shows the effect of oleate and insulin on TG and BuChE activity in Caco-2 cells. The presence of insulin had no significant effect on TG production in either unstimulated or oleate-stimulated cells. In contrast, 100 µU/ml insulin increased the activity of BuChE by about 100% in unstimulated cells.

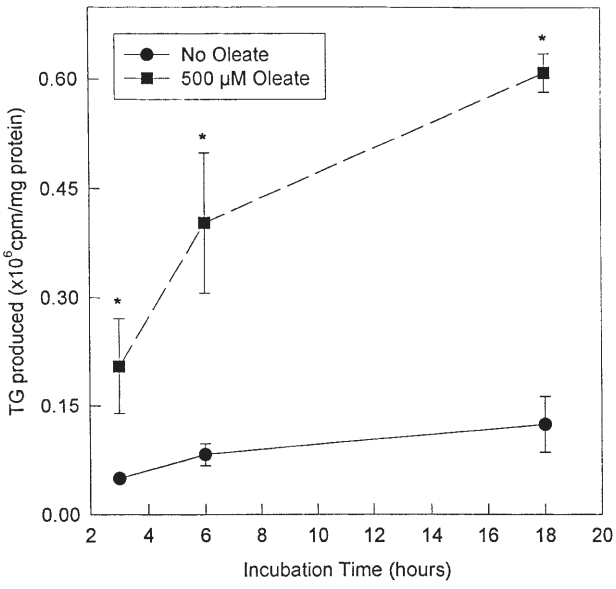

Figure 1. Production of TG Caco-2 cells. Caco-2 cells were incubated for 3, 6, or 18 h in the presence or absence of 500 µM oleate. Cell associated TG was determined by measuring the incorporation of [^{3}H]-glycerol following extraction. Data are means ± SD of three separate determinations. * $p < 0.005$ for differences between unstimulated and oleate-stimulated cells.

Table 1. Intracellular BuChE activity in Caco-2 cells.

Treatment	Cellular BuChE activity (µmol/min per milligram protein)
0 time unstimulated cells	0.0032 ± 0.0002
18 h unstimulated cells	0.0033 ± 0.0004
18 h stimulated cells	0.0031 ± 0.0002

Caco-2 cells were incubated for 0 and 18 h in the presence (stimulated) or absence (unstimulated) of 500 µM oleate. The cellular BuChE activity was determined following washing and sonication of the cellular homogenate. Results are expressed as means ± SD of three separate determinations.

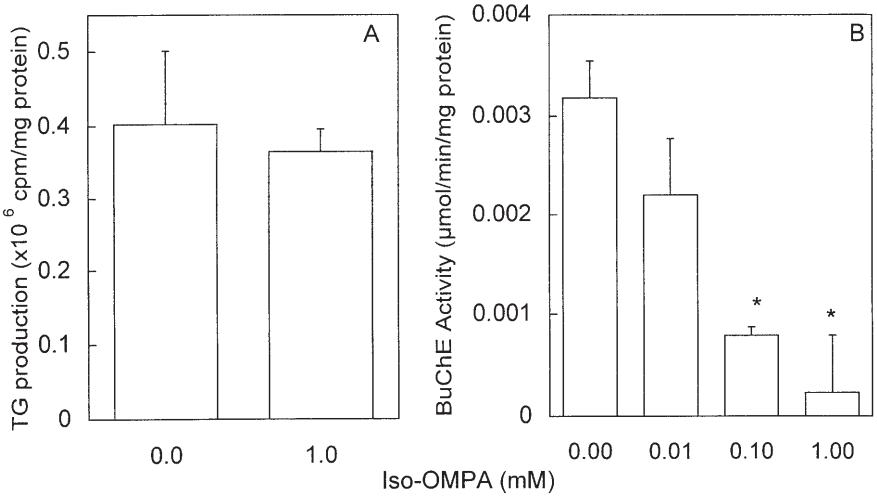

Figure 2. Effect of iso-OMPA on intracellular BuChE activity and TG production. Caco-2 cells were incubated in the presence of medium containing 500 µM oleate and increasing concentrations of Iso-OMPA for 6 h. Results are for TG production (*A*) and BuChE activity (*B*) and are expressed as means ± SD for three separate determinations. * $p < 0.005$ for differences between BuChE activity in iso-OMPA-treated and untreated cells.

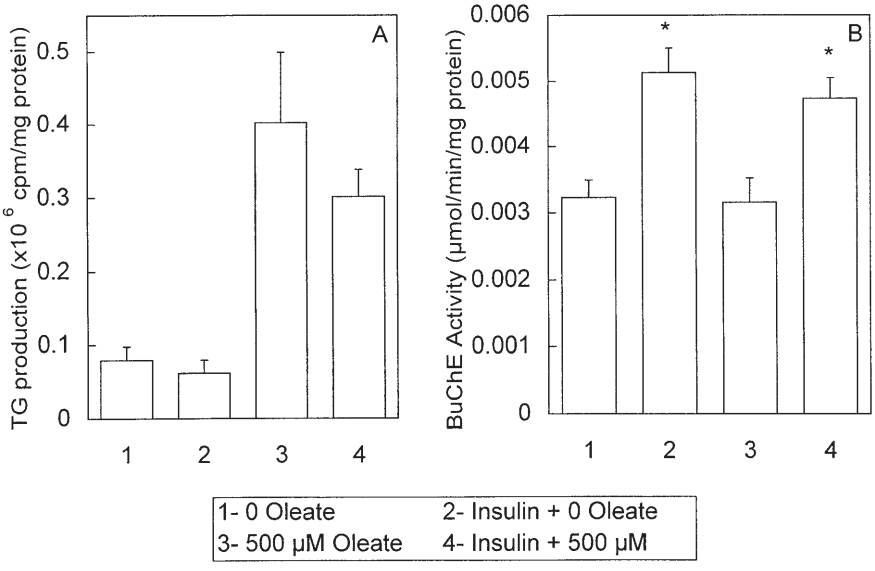

Figure 3. Effect of oleate and insulin stimulation on intracellular BuChE activity and TG production. Caco-2 cells were incubated in the presence of medium with or without 500 µM oleate or 100 µU/ ml insulin for 6 h. Results are for triglyceride production (*A*) and BuChE activity (*B*) expressed as means ± SD for three separate determinations. * $p < 0.005$ for differences between BuChE activity in insulin-stimulated and unstimulated cells.

Discussion

Patients suffering from conditions like diabetes and obesity resulting in increased production of TG and TG-rich lipoprotein usually have increased plasma levels of BuChE as well [2–7]. A number of hypotheses have been put forward to explain this including the effect of hypercaloric intake [15], hydrolysis of plasma TG and cholesterol esters by BuChE resulting in increased liver TG synthesis [6], and a direct relationship between BuChE activity and TG synthesis [16–18]. The production and secretion of TG and TG-rich lipoproteins following stimulation by free fatty acids in cultured hepatoma [11, 19] and in intestinal tumor cells [20–22] has been well documented. Although the human hepatoma cell line Hep G2 has been extensively used to study lipoprotein metabolism and this phenomenon, preliminary results have failed to show production of BuChE by these cells [K. M. Kutty, personal communication]. Others have shown evidence to the contrary [23]. For this study we used an intestinal cell line, Caco-2 cells, which produce TG-rich lipoproteins in response to oleate stimulation, to examine

the relationship between BuChE activity and TG production. We hypothesized that if BuChE and TG production occurred in a given cell type, a direct relationship may exist between them that could explain their correlation in plasma. Thus, the production of TG would be dependent on BuChE activity or vice versa.

We confirmed increased production of TG by Caco-2 cells stimulated with oleic acid. Levels of TG production were consistent with those previously reported [20–22]. However, despite an almost five-fold increase in TG production and nine-fold increase in release after 18 h with oleate stimulation, BuChE activity was unaffected. This indicates that the increased availability of substrates for TG synthesis alone is insufficient to cause increased production of BuChE activity.

The presence of BuChE activity in Caco-2 cells is a new finding. The sensitivity of human BuChE to iso-OMPA, however, is well known. Studies using HuH-7 cells showed that the cholinesterase activity secreted into a medium concentrate resembled plasma BuChE activity in its response to known inhibitors and activators of plasma BuChE [24]. That report did not comment on the levels of BuChE secreted by HuH-7 cells nor if detectable levels were present within the cells. Our studies indicate that the BuChE activity in Caco-2 cells was sensitive to iso-OMPA, but no release of enzyme activity into the cell-free medium could be detected.

When Caco-2 cell BuChE activity was inhibited by iso-OMPA, there was no effect on TG production or secretion. This result seems contradictory to previous studies. Nikkila and Kekki [25] showed inhibition of both BuChE activity and TG secretion by iso-OMPA in diabetic rats. That study, however, did not rule out toxic effects of iso-OMPA on other physiological processes that may affect lipid metabolism. Furthermore, patients with genetic abnormalities resulting in decreased levels of BuChE do not show obvious abnormalities in VLDL or TG production relative to the normal population. This suggests that other factors associated with obesity and diabetes apart from increased TG synthesis stimulate BuChE activity. Nevertheless, these results suggest that BuChE activity is not required for increased production of TG in response to oleate stimulation.

Insulin has no significant effect on TG production in oleate-stimulated Caco-2 cells [26]. This present study suggests that insulin may be one stimulus for BuChE activity. This stimulatory effect of insulin may be mediated by its receptor on Caco-2 cells [26, 27]. Previous studies have reported a positive correlation between insulin levels and BuChE activity in serum [9]. High insulin may not be the only stimulus for increased activity since induction of diabetes by destruction of pancreatic β-cells using streptozotocin also results in high levels of TG and BuChE activity [10]. These results do not support the notion suggested by others [6] that insulin may inhibit BuChE activity. The increased availability of necessary substrates for TG and BuChE production due to increased levels of feeding may be yet another stimulus [15].

In summary, the results of these studies indicate that no direct relationship exists between TG production and secretion and BuChE activity in Caco-2 cells. If these results can be applied to the liver, previous studies [3–5] showing that obese and diabetic patients have hypertriglyceridemia and increased levels of BuChE are more likely to be explained by other factors, like excess food consumption or hyperinsulinemia. Thus, increased availability of fatty acids in the liver [28] could promote the synthesis of TG, and increased availability of amino acids and/or the presence of insulin may increase BuChE activity.

Acknowledgements. We thank Ms. Millie Trask in assisting with the preparation of this manuscript. This work was supported by research funds from the Dr. Charles A. Janeway Child Health Centre at St. John's, Newfoundland.

1 Silver A. (1974) Pseudocholinesterases. In: Biology of Cholinesterase, 1st edn, pp 411–447, Neuberger A. and Tatum E. L. (eds), North-Holland, Amsterdam
2 Jain R., Kutty K. M., Huang S. N. and Kean K. (1983) Pseudocholinesterase/high-density lipoprotein cholesterol ratio in serum of normal persons and of hyperlipoproteinemics. Clin. Chem. **29:** 1031–1033
3 Chu M. I., Fontaine P., Kutty K. M., Murphy D. and Redheendran R. (1978) Cholinesterase in serum and low density lipoprotein of hyperlipidemic patients. Clin. Chim. Acta. **85:** 55–57
4 Cucuianu M., Popescu T. A. and Haragus S. (1968) Pseudocholinesterase in obese and hyperlipemic subjects. Clin. Chim. Acta. **22:** 151–155
5 Kutty K. M., Kean K. T. and Jain R. (1983) Plasma pseudocholinesterase: a potential marker for early detection of obesity. Nutr. Res. **3:** 211–215
6 Abbott C. A., Mackness M. I., Kumar S., Olukoga O. A., Gordon C., Arrol S. et al. (1993) Relationship between serum butyrylcholinesterase activity, hypertriglyceridemia in insulin sensitivity in diabetes. Clin. Sci. **85:** 77–81
7 Randell E. W., Varghese A. and Kutty K. M. (1996) Plasma cholinesterase levels are related to triglyceride levels in diabetic and obese children. Clin. Chem. **42:** S299
8 Kutty K. M., Jain R., Huang S. N. and Kean K. (1981) Serum pseudocholinesterase: high density lipoprotein cholesterol ratio as an index of risk for cardiovascular disease. Clin. Chem. Acta. **115:** 55–61
9 Kutty K. M., Jain R., Kean K. T. and Peper C. (1984) Cholinesterase activity in the serum and liver of Zucker fat rats and controls. Nutr. Res. **4:** 99–104
10 Annapurna V., Senciall I., Davis A. J. and Kutty K. M. (1991) Relationship between pseudocholinesterase in experimentally induces diabetes in rats. Diabetologia **34:** 320–324
11 Ellsworth J. L., Erickson S. K. and Cooper A. D. (1986) Very low and low density lipoprotein synthesis by the human hepatoma cell line Hep-G2: effects of free fatty acid. J. Lipid Res. **27:** 858–872
12 Dietz A. A., Rubinstein M. H. and Lubrano T. (1973) Colorimetric determination of serum cholinesterase and its genetic variants by the propionylthiocholine-dithiobis (nitrobenzoic acid) procedure. Clin. Chem. **19:** 1309–1313

13 Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. L. (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:** 265–275
14 Folch J., Lees M. and Slone-Stanley G. H. (1957) A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. **226:** 497–509
15 Kutty K. M., Huang S. N. and Kean K. T. (1981) Pseudocholinesterase in obesity: hypercaloric diet induced changes in experimental obese mice. Experimentia **37:** 1141–1142
16 Cucuianu M., Opincaru A. and Tapalaga D. (1978) Similar behaviour of lecithin: cholesterol acyltransferase and pseudocholinesterase in liver disease and hyperlipoproteinemia. Clin. Chem. Acta. **85:** 73–79
17 Popescu T. A., Fekete T., Popescu E., Bojthy I. and Laszlo M. (1976) Serum pseudocholinesterase activity during experimental fattening. Med. Intern. **14:** 71–73
18 Kutty K. M., Prabhakaran V. and Cooper A. R. (1995) Noncholinergic function of cholinesterases in enzymes of the cholinesterase family. In: Enzymes of the Cholinesterase Family, pp. 424–426, Quinn D. M., Balasubramanian A. S., Doctor B. P. and Taylor P. (eds.), Plenum, New York
19 Arrol S., Mackness M. I., Laing I. and Durrington P. N. (1991) Lipoprotein secretion by the human hepatoma cell line HepG2: differential rates of accumulation of apolipoprotein and lipoprotein lipid in tissue culture in response to albumin, glucose and oleate. Biochim. Biophys. Acta **1086:** 72–80
20 Hughes T. E., Sasak W. V., Ordovas J. M., Forte T. M., Samon-Fava S. and Schaefer E. J. (1987) A novel cell line (Caco-2) for the study of intestinal lipoprotein synthesis. J. Biol. Chem. **262:** 3762–3767
21 Hughes T. E., Ordovas J. M. and Schaefer E. J. (1988) Regulation of intestinal lipoprotein B synthesis and secretion by Caco-2 cells. J. Biol. Chem. **263:** 3425–3431
22 Field F. J., Albright E. and Mathur S. N. (1988) Regulation of triglyceride-rich lipoprotein secretion by fatty acids in Caco-2 cells. J. Lipid. Res. **29:** 1427–1436
23 Osada J., Lopez-Miranda J. and Ordovas J. M. (1994) The human hepatoma cell line, HepG2, secretes functional cholinesterase. Biochem. Mol. Biol. Int. **33:** 1099–1105
24 Hada T., Yamamoto T., Imanishi H., Takahashi S., Amuro Y., Higashino K. et al. (1987) Novel cholinesterase expression in the HuH-7 cell line. Tumor. Biol. **8:** 3–8
25 Nikkila E. A. and Kekki M. (1973) Plasma triglyceride transport kinetics in diabetes mellitus. Metabolism **22:** 1–22
26 Jiao S., Moberly J. B., Cole T. G. and Schonfeld G. (1989) Decreased activity of Acyl-CoA: cholesterol acyltransferase by insulin in human intestinal cell line Caco-2. Diabetes **38:** 604–609
27 MacDonald R. S., Thornton W. H. and Bean T. L. (1993) Insulin and IGF-1 receptors in a human intestinal adenocarcinoma cell line (Caco-2): regulation of Na^+ glucose transport across the brush border. J. Receptor. Res. **13:** 1093–1113
28 Kean K. T., Kutty J. M., Huans S. N. and Jain R. (1986) A study of pseudocholinesterase in experimental obesity. J. Am. Coll. Nut. **5:** 253–261