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Inhibition of neutral cholesteryl ester hydrolase by a naturally occurring cytosolic protein in macrophages

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The soluble fraction from a number of macrophage cell lines has been shown to contain a protein(s) that inhibits neutral cholesteryl ester hydrolase. The inhibition is dependent on the concentration of soluble protein used, and the efficiency of the inhibitor can be influenced by the inclusion of oleate and an inhibitor of ACAT. It is suggested that the presence of this material indicates that the macrophage contains a means of negatively controlling the activity of the hydrolytic phase of the cholesterol/cholesteryl ester cycle.

Cholesteryl ester hydrolase; Inhibition; Macrophage; Atherosclerosis

1. INTRODUCTION

Atherosclerosis is characterised by the appearance of cholesteryl ester-laden foam cells that are derived from macrophages within the intimal layer of the artery [1–3]. The mechanism by which these monocyte-derived macrophage foam cells accumulate excess lipid in the artery wall in vivo has not been determined although macrophage cell cultures, in vitro, have been shown to accumulate cholesterol via both normal low density lipoprotein (LDL) receptors and the 'scavenger' receptor pathway [4,5] and to store the sterol in the esterified form in lipid droplets [6]. The cholesteryl esters in the cytoplasmic droplets are not inert but undergo a cycle of continual hydrolysis and re-esterification [4]. The rate-limiting enzyme in the esterification phase of the cycle is acyl-coenzyme A:cholesteryl acyl *O*-transferase (ACAT) [4,5,7], while a cytoplasmic neutral cholesteryl ester hydrolase (CEH) is believed to be the enzyme responsible for the hydrolytic phase [4,8]. In at least two macrophage-like murine tumour cells, the activity of neutral CEH has been shown to be activated by cAMP-dependent protein kinase [9] in a manner similar to that observed for hormone-sensitive lipase to which it is immunologically identical [10]. We have recently demonstrated that rat liver cells contain a cytoplasmic protein that is a highly active inhibitor of the neutral CEH [11,12]. We show here that a number of macrophage cell lines also contain a cytosolic protein inhibitor of neutral CEH and that the activity of this material can be increased under conditions that are reportedly characterised by enhanced accumulation of the sterol ester [13].

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2. MATERIALS AND METHODS

2.1. *Materials*

Microsomes were prepared from the mid-lactating mammary glands of Wistar rats [11]. Cholesteryl [^{3}H]oleate was prepared as previously described [11]. Compound 58-035 (3-[decyldimethyl-silyl]-*N*-[2-(4-methylphenyl)-1-phenylethyl]propanamide) was generously provided by Sandoz Inc., East Hanover, NJ, USA. All other biochemicals were obtained from Sigma or BCL.

2.2. *Preparation of macrophage cytosols*

The macrophage cell lines J111, J774A1, J774A2 and P388D1, were grown as monolayer cultures in Fischer's medium (Gibco) supplemented with 2 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin and 10% newborn calf serum. Cell density was maintained between 4×10^5 and 2×10^6 cells/ml. Where indicated the supernatants from confluent cultures were removed and replaced with an equal volume of fresh culture medium containing 100 μg/ml oleate with and without the addition of 58-035 (0.5 μg/ml in dimethylsulphoxide).

After incubation for a further 24 h the supernatants were removed and the cells washed 3 times with sterile Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS). The cell monolayer was detached using 0.02% trypsin and harvested by centrifugation at $500 \times g$ for 5 min. The cell pellet was washed 3 times with PBS before suspension in 50 Mm Tris-50 mM MES, pH 7.2, containing 1 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, and 1 μg/ml of leupeptin, pepstatin and antipain. The cells were disrupted by sonicating for 4×30 s on ice, using an exponential microtip coupled to a Kontes 50 watt micro ultrasonic cell disrupter. Cell debris and membranes were removed by centrifugation for 10 min at $178.000 \times g$ (Beckman Airfuge, A100-30 rotor) and the soluble extract used fresh or stored in liquid nitrogen. Aliquots of the soluble extracts were also used to prepare an acetone-diethyl ether powder [11], which were then re-extracted in an equal volume of the homogenization buffer. Aliquots of the extracts were heated at 95°C for 5 min and the precipitated protein removed by centrifugation for 5 min at $10,000 \times g$. Further aliquots were passed through a column (0.8×3 cm) of Sephadex G15, equilibrated in the homogenization buffer.

2.3. *Enzyme activity*

Mammary microsomal neutral CEH activity was determined in the presence and absence of aliquots of the soluble extracts at pH 7.2 against cholesteryl [^{3}H]oleate in ethanolic suspension [11]. Protein was determined by the method of Bradford [13].

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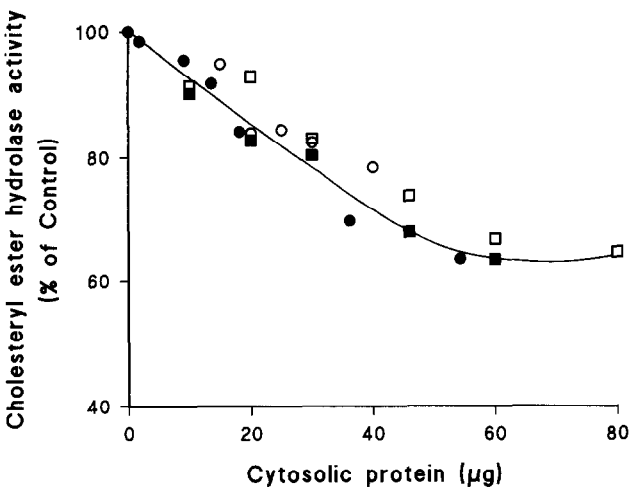

Fig. 1. Variation of the inhibitory activity with concentration of cytosolic protein. Mammary microsomes (100 μg) were assayed, in triplicate, for cholesteryl ester hydrolase activity in the presence of varying amounts of cytosolic protein obtained from P388D1 cells grown in culture. The results shown are from four different cultures. The control (100%) value was 357.9 ± 3.9 pmol cholesteryl oleate hydrolysed per min/mg of protein.

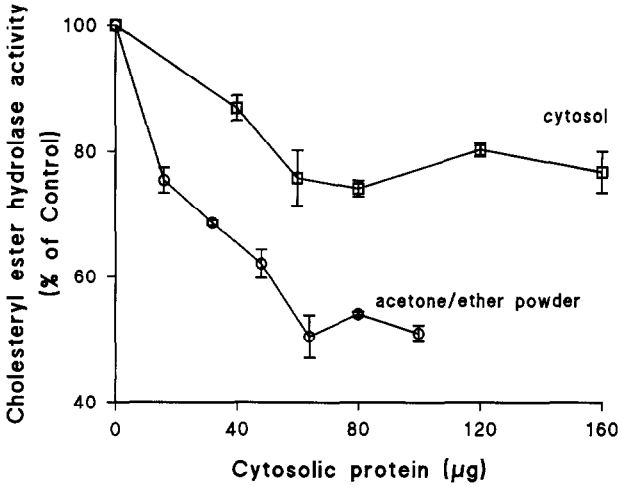

Fig. 2. Inhibition of neutral cholesteryl ester hydrolase: effect of preparing an acetone-diethyl ether powder from the cellular cytosols. An aliquot of the cytosol from J774A2 cells was converted to an acetone-diethyl ether powder before being re-extracted into buffer and added to the assay of mammary microsomal CEH activity. The control (100%) value was 369.2 pmol/min/mg protein. The results are the means ± S.E.M. of triplicate observations.

3. RESULTS AND DISCUSSION

When the mammary microsomal CEH was assayed in the presence of an aliquot of the cytosolic fraction from the J111 macrophage cells the hydrolytic activity of the enzyme was inhibited (Table I). Each of the cell lines J111, J774A1, J774A2 and P388D1, contained inhibitory protein factor(s) that behaved in a similar way (Table I). The amount of inhibitory protein in the cultures was variable, and similar amounts of cytosolic protein from the four cell lines gave different degrees of inhibition. The cytosols from the J774A2 cells contained more inhibitory activity than those from the J774A1 cells. Interestingly J774A2 cells are a variant of the J774A1 line which are able to store a greater amount of cholesteryl ester than the J774A1 cells [14].

The inhibitory effect was dependent on the concentration of cytosolic protein used (Figs. 1 and 3) but total inhibition of the CEH activity was not achieved. The inhibitory protein was destroyed by heating (Table I) and by incubation with trypsin (results not shown) but its activity was unaffected by passage through Sephadex G15 (Table I). The macrophage inhibitory protein survived the preparation of an acetone-diethyl ether powder (Fig. 2) and this process appeared to enhance the specific activity. Similar results were obtained for all of the four cell lines, but only the selected results are shown.

Table I

Inhibition of neutral cholesteryl ester hydrolase by cytosolic protein in macrophage cell lines

Cell line	Cytosolic protein	Treatment	CEH activity (pmol/min/mg)	Inhibition (%)
J111	76 μg	none	171.1 ± 4.7	53.6
J111	76 μg	Sephadex	157.4 ± 13.8	57.2
J111	–	heated	344.4 ± 6.5	6.5
P388D1	60 μg	none	240.4 ± 3.8	34.7
J774A1	50 μg	none	328.9 ± 7.1	10.7
J774A2	50 μg	none	301.6 ± 1.9	18.1

The indicated macrophage cell lines were grown in culture and aliquots of the cytosolic fraction were included in the assay for the mammary microsomal neutral cholesteryl ester hydrolase. Full experimental details of the methodology and of the Sephadex G15 and heat treatments are given in section 2. The results are the means ± S.E.M. of 3–9 determinations. The uninhibited neutral cholesteryl ester hydrolase activity of the mammary microsomes was 368.3 ± 7.2 pmol of cholesteryl oleate hydrolysed per min/mg of microsomal protein.

Table II

The inhibition of mammary neutral cholesteryl ester hydrolase by cytosolic protein from J774A2 macrophages: influence of oleic acid and the ACTA inhibitor 58-035

Cytosolic protein	Culture condition	CEH activity (pmol/min/mg)	Inhibition (%)
none	–	310.6 ± 1.6	0
26.4 μg	Oleic	206.1 ± 9.2	33.6
21.0 μg	Oleic + 58035	260.4 ± 5.7	17.2

J774A2 macrophage cells were cultured in the presence of oleic acid (100 μg/ml) with and without the ACAT inhibitor, 58–035 (0.5 μg/ml). The cellular soluble protein fractions were converted to acetone-diethyl ether powders before re-extraction and inclusion in the assay. The results are the means±S.E.M. of triplicate observations.

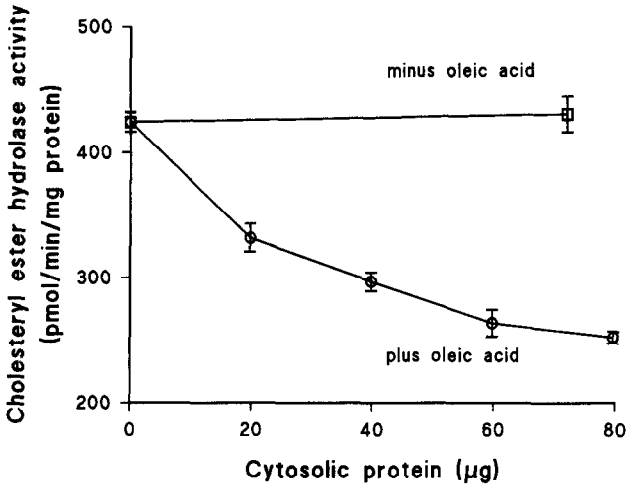

Fig. 3. The influence of oleate in cell culture medium on the efficiency of the inhibitor of neutral cholesteryl ester hydrolase. J774A1 cells were cultured for 24 h in medium with and without the addition of 100 μg oleate/ml. The influence of the cytosol fractions on mammary microsomal CEH activity was determined. The results are the means ± SEM of triplicate observations.

Incubation of J774 macrophages with oleic acid, which has been shown to lead to an increase in the accumulation of cholesteryl esters in the cells [15], enhanced the activity of the inhibitory material (Fig. 3). When J774A2 cells, grown in the presence of oleate to enhance cholesterol accumulation, were incubated in the presence of the ACAT inhibitor, 58035, the activity the inhibitory material was decreased (Table II). Treatment of J774 macrophages with the inhibitor 58–035 has been shown to markedly diminish the total cholesterol uptake/synthesis in the cell [16]. The reduction in inhibitor efficiency can then be rationalised on the basis that increased CEH activity was required to maintain the cellular pool of free cholesterol.

All the data presented here strongly support the conclusion that the soluble cell fraction of these macrophages contain naturally occurring protein inhibitor(s) of the mammary microsomal neutral CEH which we have shown to be immunologically identical to the hormone-sensitive lipase of bovine adipose tissue [17], which is identical to the macrophage neutral CEH [10]. We can thus assume that the macrophage CEH is also susceptible to inhibition in this manner. The presence of this inhibitory protein(s) indicates that, in addition to the reported cAMP-dependent stimulation of this enzyme [9], the cells also contain a mechanism for negatively regulating its activity. This dual regulation emphasises the importance of this enzyme in maintaining the balance between cholesterol and its esters.

Elevated plasma fatty acids are a risk factor associated with atherosclerosis so it is particularly interesting that the activity of this inhibitory material could be varied by the inclusion of oleate. This would lead to increased cholesteryl ester accumulation and suggests that factors, which vary the activity of the inhibitor, may play a role in the development of the disease.

Since the bulk of free cholesterol is contained in the plasma membrane [7,18,19] variations in the cellular balance of cholesterol could affect both plasma membrane sterol content and fluidity, with consequent effects on the secretion of factors that may contribute to intimal thickening. Thus, although this inhibitory protein presents a novel negative control mechanism for the activity of the neutral CEH, its presence may be of great importance to the cell for the maintenance of membrane integrity.

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