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Hormone-sensitive lipase is responsible for the neutral cholesterol ester hydrolase activity in macrophages

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Anti-hormone-sensitive lipase (HSL) immunoglobulin selectively immunoprecipitates a single 84 kDa ^{32}P-phosphoprotein from macrophage homogenates previously phosphorylated by cyclic AMP-dependent protein kinase in the presence of [γ-^{32}P]ATP-Mg. This immunoglobulin also completely removes the neutral cholesterol ester hydrolase activity from macrophage homogenates. These data demonstrate that HSL is responsible for the neutral cholesterol ester hydrolase activity in macrophages and hence plays a key role in cholesterol metabolism in these cells.

Hormone-sensitive lipase; Cholesterol ester hydrolase; Macrophage; Atherosclerosis

1. INTRODUCTION

Macrophages are believed to be precursors of the lipid-laden foam cells present in atherosclerotic plaques [1]. Macrophages maintained in tissue culture can be induced, by incubation with low-density lipoprotein, to accumulate large stores of esterified cholesterol and take on the appearance of foam cells [2]. Furthermore, the cholesterol esters in smooth muscle foam cells have been shown to be derived from recycled inclusions taken up from lysed macrophages [3]. Since the accumulation of cholesterol esters in macrophages has such implications, it is clearly of great importance to elucidate the mechanisms whereby they are stored and mobilized in this cell type. Macrophages internalise LDL in endocytotic vesicles which are passed onto lysosomes where the cholesterol esters are degraded by an acid hydrolase. This liberates free cholesterol which leaves the lysosome and is either excreted, passing to HDL which acts as acceptor, or re-esterified in the cytoplasm by ACAT.

Stored cholesterol ester can be hydrolysed subsequently by a cytoplasmic neutral cholesterol ester hydrolase and passed to HDL or, in the absence of HDL, the free cholesterol is retained in the cell and re-esterified by ACAT; about 50% of the stored cholesterol esters are hydrolysed and re-esterified each day in this cycle [2]. During conditions of cholesterol ester accumulation the rate of esterification must clearly exceed that of hydrolysis of the esters.

A neutral cholesterol ester hydrolase activity has been demonstrated in cell-free extracts of macrophages and shown to be activated by cyclic AMP-dependent protein kinase [4], consistent with regulation of this enzyme by phosphorylation. It has been demonstrated previously that HSL has cholesterol ester hydrolase activity [5] and that, in addition to its role in lipolysis [6], HSL is responsi-

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Abbreviations: ACAT, acyl CoA:cholesterol acyltransferase; HDL, high-density lipoprotein; HSL, hormone-sensitive lipase; LDL, low-density lipoprotein

Enzymes: hormone-sensitive lipase or triacylglycerol lipase (EC 3.1.1.3); acyl CoA:cholesterol acyltransferase (EC 2.3.1.26); cyclic AMP-dependent protein kinase (EC 2.7.1.37)

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ble for the cholesterol ester hydrolase activity in steroidogenic tissues [7,8]; furthermore, its cholesterol ester hydrolase activity can be activated by cyclic AMP-dependent protein kinase-mediated phosphorylation [9]. In this report we present direct evidence that HSL is responsible for the neutral cholesterol ester hydrolase activity in macrophages, providing a possible mechanism for hormonal or therapeutic control of intracellular levels of cholesterol esters.

2. MATERIALS AND METHODS

2.1. *Materials*

HSL was purified from bovine perirenal adipose tissue as in [10]. The catalytic subunit of cyclic AMP-dependent protein kinase was purified to homogeneity from bovine adipose tissue [11]. Antiserum against bovine adipose tissue HSL was raised in a New Zealand White rabbit and an immunoglobulin fraction prepared as in [12]. [γ-^{32}P]ATP was from Amersham International, hexokinase was from Boehringer, Pansorbin from Calbiochem and trypsin (tosyl-phenylalanyl chloromethyl ketone treated) from Worthington. Sources of substrates, protease inhibitors and the non-ionic detergent $C_{13}E_{12}$ were as described in [10]. Fuji RX X-ray film was used for autoradiography.

2.2. *Preparation of macrophage homogenate*

The mouse macrophage cell-line WEHI (supplied kindly by Dr C.G. Brooks, University of Newcastle upon Tyne) was maintained in RPMI-1640 medium (Gibco), containing 10% fetal bovine serum (Sigma), 50 U/ml penicillin, 50 μg/ml streptomycin, 3 mM glutamine and 50 μM 2-mercaptoethanol. Cells were harvested by centrifugation at 500 × *g* for 5 min and the cell pellet resuspended at a final concentration of approx. 5 × 10^6 cells/ml in 5 mM imidazole, pH 7.0, containing 0.1 mM benzamidine-HCl, 1 mM dithiothreitol, 30% glycerol, 0.2% $C_{13}E_{12}$, 50 mM NaCl, 5 μg/ml leupeptin and 1 μg/ml pepstatin. The WEHI cells were disrupted by sonicating for 4 × 30 s on ice, using an exponential microtip coupled to a Dawe Type 7533A ultrasonic generator. Cell debris was removed by centrifugation at 10000 × *g* for 5 min and the soluble extract used fresh or stored at −70°C until use.

2.3. *Enzyme activity*

Neutral cholesterol ester hydrolase activity was assayed at pH 7.0 against cholesterol [^{3}H]oleate in ethanolic suspension [13]. 1 unit of activity catalyses the release of 1 μmol fatty acid per min. Protein was determined by the method of Bradford [14].

2.4. *Phosphorylation of purified HSL and macrophage homogenate*

Prior to phosphorylation, purified bovine HSL was transferred into 5 mM imidazole-HCl (pH 7.0), containing 1 mM dithiothreitol, 30% (w/v) glycerol, 50 mM NaCl, 5 μg/ml leupeptin and 1 μg/ml pepstatin [12]. Phosphorylation of the purified HSL and macrophage homogenate was performed in the presence of 0.1 mM [γ-^{32}P]ATP (5000–10000 dpm/pmol), 1 mM $MgCl_2$ and 3 μM catalytic subunit of cyclic AMP-dependent protein kinase, as described in [15].

2.5. *Preparation of immunoprecipitates*

Immunoprecipitates were prepared by incubating either the ^{32}P-phosphorylated purified HSL or macrophage extract with anti-HSL immunoglobulin as in the legend to fig.1, following the procedure detailed in [12]. Controls were incubated with normal rabbit serum, then treated in an identical manner. The immunoprecipitates were analysed by SDS-PAGE [16] followed by autoradiography. Tryptic digestion of the immunoprecipitated 84 kDa phosphoprotein and separation of the phosphopeptides by reverse-phase HPLC was carried out as in [15]. The phosphopeptides were also analysed by high-voltage electrophoresis at pH 1.9 and autoradiography [12].

Non-phosphorylated macrophage homogenate was incubated with anti-HSL immunoglobulin as in the legend to fig.2. Following treatment with Pansorbin and centrifugation to precipitate the immune complexes [12], the supernatants were assayed for cholesterol ester hydrolase activity. Controls were performed using normal rabbit serum.

3. RESULTS AND DISCUSSION

When macrophage extracts were incubated with the catalytic subunit of cyclic AMP-dependent protein kinase in the presence of [γ-^{32}P]ATP-Mg, a large number of polypeptides became phosphorylated (fig.1). Treatment of the phosphorylated extracts with anti-HSL immunoglobulins led to immunoprecipitation of a single phosphorylated polypeptide of approx. 84 kDa (fig.1), the same size as the HSL polypeptide from several other sources [5,10]. Immunoprecipitation of the 84 kDa ^{32}P-phosphoprotein was blocked in a competitive manner by the presence of increasing amounts of non-radioactive HSL from adipose tissue. The site on adipose tissue HSL which is phosphorylated by cyclic AMP-dependent protein kinase has recently been characterised and sequenced [15]; when tryptic phosphopeptides from the macrophage 84 kDa ^{32}P-phosphoprotein and bovine HSL were subjected to reverse-phase HPLC, two major peaks of radioactivity were recovered in each case, corresponding in elution positions to phosphopeptides T1 and T2 described previously [15]. When the tryptic ^{32}P-phosphopeptides were analysed by high-voltage electrophoresis and autoradiography, the same electrophoretic pattern was observed for ^{32}P-phosphopeptides derived from the macrophage 84 kDa protein and adipose tissue HSL (results not shown).

Taken together, the above data strongly support

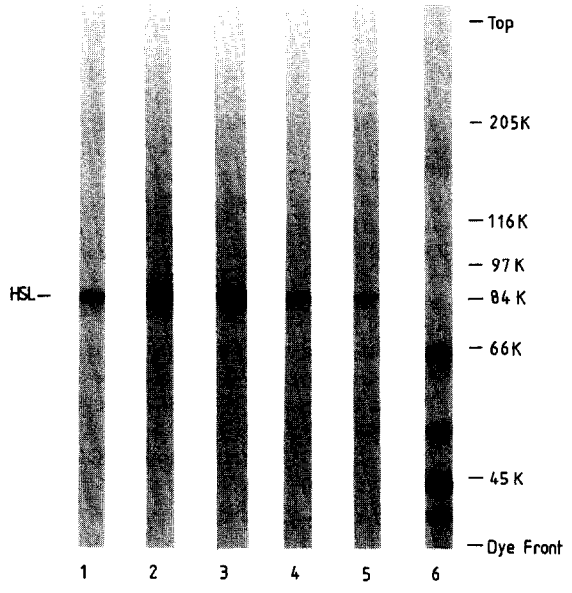

Fig.1. Immunoprecipitation of phosphoproteins from ^{32}P-phosphorylated macrophage extracts. Macrophage extract (100 μg) was incubated for 30 min at 30°C with the catalytic subunit of cyclic AMP-dependent protein kinase (3 μM), 1 mM $MgCl_2$ and 0.1 mM [γ-^{32}P]ATP (5000–10000 dpm/pmol), as was purified HSL (1.25 μg). Phosphorylation was terminated by the addition of 0.1 vol. of 1 M glucose and 0.01 vol. of 10 mg/ml hexokinase. Immunoprecipitates (lanes 1–5) were prepared from the ^{32}P-phosphorylated samples using anti-HSL immunoglobulin (375 μg) [12]; in lanes 3–5, unlabelled adipose tissue HSL was added to the ^{32}P-labelled macrophage extract prior to addition of anti-HSL immunoglobulin, as indicated. Samples were analysed by SDS-PAGE and autoradiography. Lanes: 1, adipose tissue HSL; 2, macrophage extract; 3, 4 and 5, macrophage extract + 1.0, 10 and 50 ng unlabelled adipose tissue HSL, respectively; 6, ^{32}P-phosphorylated macrophage extract (25 μg) without immunoprecipitation.

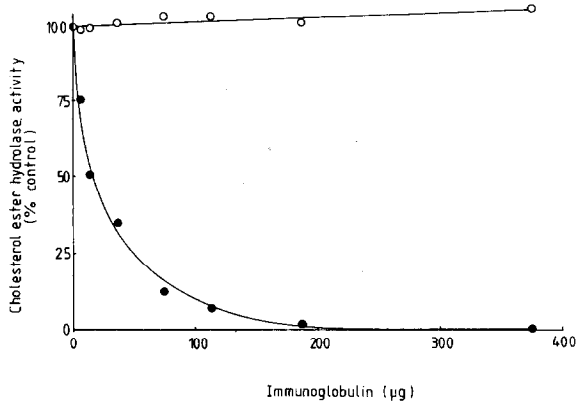

Fig.2. Effect of anti-HSL immunoglobulin on the neutral cholesterol ester hydrolase activity in WEHI macrophage extracts. Macrophage extract (50 μl containing approx. 30 μg protein) was incubated overnight at 4°C with the indicated amounts of anti-HSL immunoglobulin (●) or non-immune immunoglobulin (○); all incubations contained the same final concentration of protein (400 μg), achieved by the addition of an appropriate amount of bovine serum albumin (BSA). The immune-complexes were precipitated as in [12] and the supernatants assayed against cholesterol [^3H]oleate. Activity is expressed as a percentage of that observed in the presence of 400 μg BSA alone.

the conclusion that HSL is present within macrophages and suggest that it may contribute to the neutral cholesterol ester hydrolase activity in these cells. The neutral cholesterol ester hydrolase in WEHI cell extracts was found to have a specific activity of 0.7 mU/mg, comparable to values reported by Khoo et al. [4]; activity was linear with respect to the concentration of protein in the extract (not shown). To quantitate the contribution of HSL to this activity, anti-HSL immunoglobulin was incubated with the extracts and the cholesterol ester hydrolase activity assayed in the resultant immunosupernatants. As can be seen in fig.2, the cholesterol ester hydrolase activity could be precipitated completely by the anti-HSL immunoglobulin, demonstrating that HSL is responsible for most, if not all, of the neutral cholesterol ester hydrolase activity in these extracts.

Thus, all the data presented here strongly support the conclusion that HSL is responsible for the neutral cholesterol ester hydrolase activity in macrophages. This is consistent with the previous observation that this cholesterol ester hydrolase activity is activated by cyclic AMP-dependent protein kinase [4]. The ability of the macrophage to mobilize cholesterol ester stores may therefore be under regulation via the levels of intracellular cyclic AMP. The mechanism whereby this activation could be facilitated in vivo is unknown, however certain agents such as adenosine [17] and several prostaglandins, including PGE_2 and PGI_2 [18], have been reported to increase intracellular cyclic AMP levels in macrophages. Raised levels of cyclic AMP would be expected to lead to phosphorylation of HSL by cyclic AMP-dependent protein kinase and potentially to increased mobilization of macrophage cholesterol ester stores due to activation of HSL. However this has yet to be demonstrated, but identification of the protein responsible for cholesterol ester

breakdown in these cells now makes this feasible. The recent finding that activation of HSL by cyclic AMP-dependent protein kinase can be blocked by prior phosphorylation of HSL by the AMP-activated protein kinase in vitro [19] may also have implications for cholesterol metabolism as WEHI cells contain significant levels of AMP-activated protein kinase (Davies, S., Small, C.A., Yeaman, S.J. and Hardie, D.G., unpublished). Clearly it is now possible to study regulation of the activity of HSL in both normal macrophages and in cells loaded with cholesterol esters.

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