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Phosphorylation and activation of hormone-sensitive lipase in isolated macrophages

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Hormone-sensitive lipase (HSL) is responsible for the neutral cholesterol ester hydrolase activity in macrophages. Incubation of intact WEHI macrophages or mouse peritoneal macrophages leads to phosphorylation of HSL, which is increased by incubation with either dibutyryl cyclic AMP and 3-isobutyl-1-methylxanthine or okadaic acid. Correspondingly, these agents also activate neutral cholesterol ester hydrolase activity in intact WEHI cells. Regulation of mobilisation of esterified cholesterol in macrophages may be of antiatherogenic value, which this model system now allows us to investigate further.

Hormone-sensitive lipase; Protein phosphorylation; Macrophage; Cholesterol metabolism

1. INTRODUCTION

The lipid-laden foam cells characteristic of atherosclerotic plaques are derived, at least in part, from macrophages which have become overloaded with cholesterol esters. Hence, an understanding of the mechanisms whereby these cells accumulate esterified cholesterol is of importance in understanding the pathogenesis of atherosclerosis [1]. The cholesterol ester droplets in the cytoplasm of macrophages are in a dynamic state, undergoing a constant cycle of hydrolysis by a cytosolic cholesterol ester hydrolase and re-esterification by acyl CoA: cholesterol acyl transferase (ACAT) [2]. The regulation of both ACAT and cytoplasmic cholesterol ester hydrolysis may therefore play an important role in the control of esterified cholesterol levels in the macrophage.

We have previously identified the neutral cholesterol ester hydrolase in cell-free extracts of the WEHI macrophage cell-line as hormone-sensitive lipase (HSL) [3], a multifunctional hydrolase with activity against several lipid substrates including triacylglycerol and cholesterol esters [4]. A key feature of HSL is its ability to be activated by phosphorylation by cyclic AMP-dependent protein kinase [5–7]. Consistent with this, Goldberg and Khoo have recently demonstrated activation of neutral cholesterol ester hydrolase activity in cell-free extracts of P388D$_1$ macrophages by cyclic AMP-dependent protein kinase, and in intact cells by elevation of intracellular cyclic AMP levels [8]. In this report we extend these previous findings and demonstrate that HSL is present in mouse peritoneal macrophages, that it can be phosphorylated within intact macrophages and that phosphorylation is associated with increased cholesterol ester hydrolase activity.

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Enzymes: hormone-sensitive lipase or triacylglycerol lipase, EC 3.1.1.3; cyclic AMP-dependent protein kinase, EC 2.7.1.37

2. MATERIALS AND METHODS

2.1. *Materials*

HSL and the catalytic subunit of cyclic AMP-dependent protein kinase were purified from bovine perirenal adipose tissue as in [9]. Antiserum against bovine adipose tissue HSL was raised in a New Zealand White rabbit and an immunoglobulin fraction prepared as in [10]. [γ-^{32}P]ATP was from ICN, [^{32}P]orthophosphate was from Amersham, dibutyryl cyclic AMP and 3-isobutyl-1-methylxanthine were from Sigma, and Pansorbin was from Calbiochem. Okadaic acid was a generous gift from Prof. P. Cohen (University of Dundee). Sources of substrates, protease inhibitors and the non-ionic detergent $C_{13}E_{12}$ were as described in [9]. Amersham Hyperfilm-MP X-ray film was used for autoradiography.

2.2. *Macrophage preparation and incubation*

The mouse macrophage cell-line WEHI (kindly supplied by Dr C.G. Brooks, University of Newcastle upon Tyne) was maintained in RPMI-1640 medium (Northumbria Biologicals), containing 10% foetal calf serum (Sigma), 50 U/ml penicillin, 50 U/ml streptomycin, 3 mM glutamine (Gibco) and 50 μM 2-mercaptoethanol (Sigma). Cells were harvested by centrifugation at 200 × *g* for 10 min and the cell pellet resuspended at a final concentration of approx. 5 × 10^7 cells/ml in Eagle's minimum essential medium (EMEM) (Northumbria Biologicals) containing 10% foetal calf serum, 50 U/ml penicillin, 50 U/ml streptomycin, 3 mM glutamine and 50 μM 2-mercaptoethanol (medium A).

Peritoneal macrophages were harvested from unstimulated female BALB/c mice (4–6 weeks old) in Dulbecco's phosphate-buffered

saline (Gibco). The cells from 6–12 mice (approx. 2×10^8 cells per mouse) were pooled, collected by centrifugation at $200 \times g$ for 10 min and washed three times in EMEM. The washed cells were resuspended in medium A at a final concentration of approx. 5×10^7 cells/ml.

In experiments measuring phosphorylation of intracellular proteins, 50 μl of either WEHI cells or mouse peritoneal macrophages were preincubated for 2 h with 500 μCi/ml, 0.8 mM [^{32}P]orthophosphate to allow equilibration of the radioactive phosphate with intracellular ATP. ^{32}P-phosphorylation of intracellular proteins was then stimulated by the addition of either 1.5 mM dibutyryl cyclic AMP and 100 μM 3-isobutyl-1-methylxanthine, 1 μM okadaic acid, or a mixture of all three. After 15 min, cells were harvested by centrifugation at $10000 \times g$ for 1 min and washed twice in EMEM. The cell pellet was resuspended in 1.0 ml of 20 mM Tris-HCl, pH 7.0, containing 20 mM NaP_2O_7, 1% (v/v) Triton X-100, 1% (w/v) SDS, 2% (w/v) sodium deoxycholate, 1 mM EDTA, 1 mM EGTA, 5 mM NaF, 20 μg/ml leupeptin, 10 μM TLCK, 0.1 mM PMSF and 1 mM dithiothreitol and the cell pellet disrupted by sonication for 2×15 s on ice, using an exponential microtip coupled to a Dawe type 7533A ultrasonic generator. Cell debris was removed by centrifugation at $10000 \times g$ for 5 min. Immunoprecipitates were prepared from the soluble extract using anti-HSL immunoglobulin [3] and analysed by SDS-PAGE [11] followed by autoradiography.

2.3. *Assay of cholesterol ester hydrolase activity*

In experiments measuring activation of neutral cholesterol ester hydrolase activity, 1.0 ml suspensions of WEHI macrophages were incubated with either 1.5 mM dibutyryl cyclic AMP and 100 μM 3-isobutyl-1-methylxanthine, 1.0 μM okadaic acid, or a mixture of all three. After various times, cells were harvested by centrifugation at $10000 \times g$ for 1 min, washed in Dulbecco's phosphate-buffered saline and the cell pellet resuspended in 0.5 ml of 5 mM imidazole-HCl, pH 7.0, containing 0.1 mM benzamidine-HCl, 1 mM dithiothreitol, 30% (w/v) glycerol, 0.2% (w/v) $C_{12}E_{12}$, 50 mM NaCl, 5 mM NaP_2O_7, 20 mM EDTA, 5 μg/ml leupeptin and 1 μg/ml pepstatin. The WEHI cells were disrupted by sonication as described previously and the

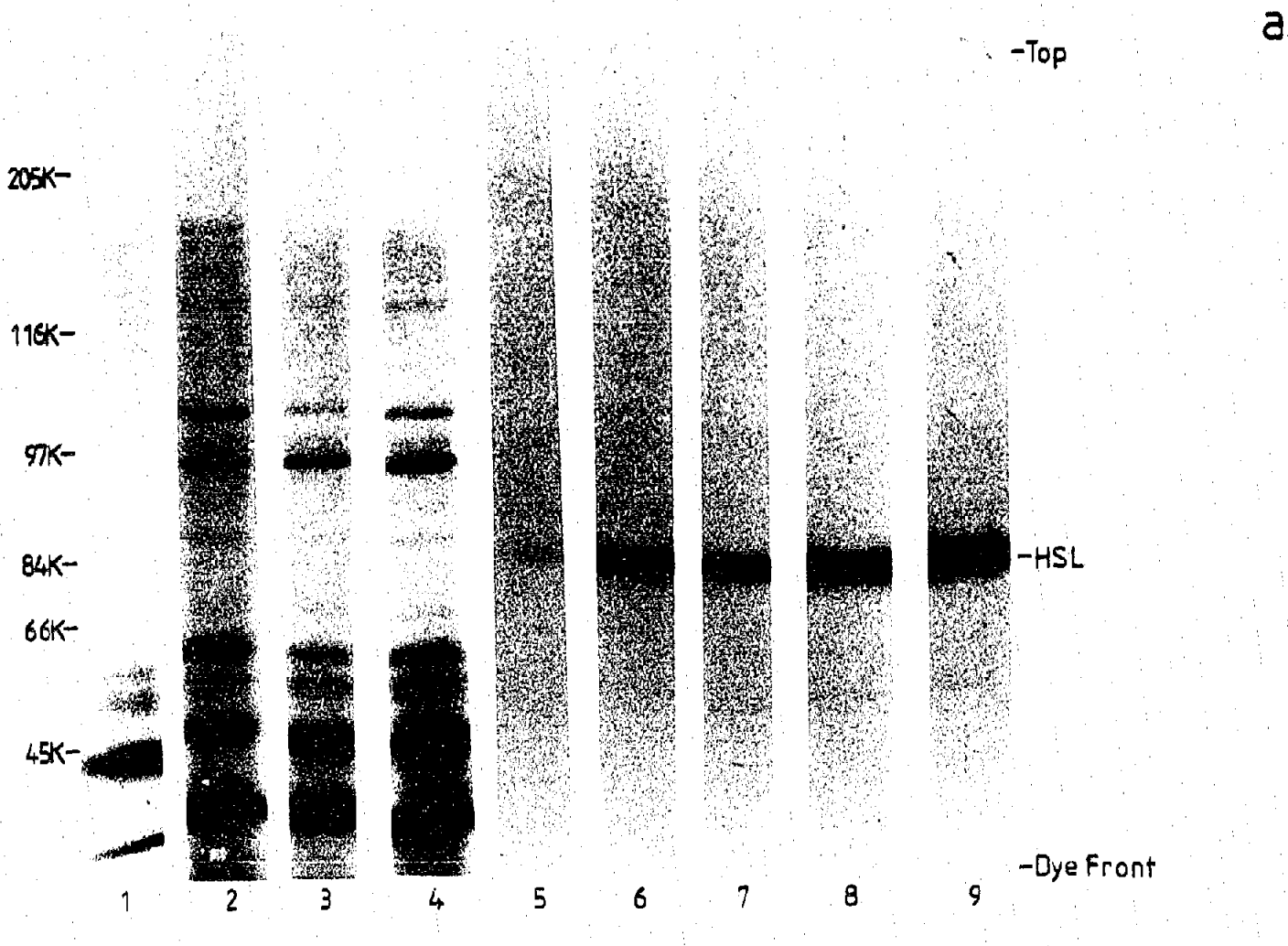


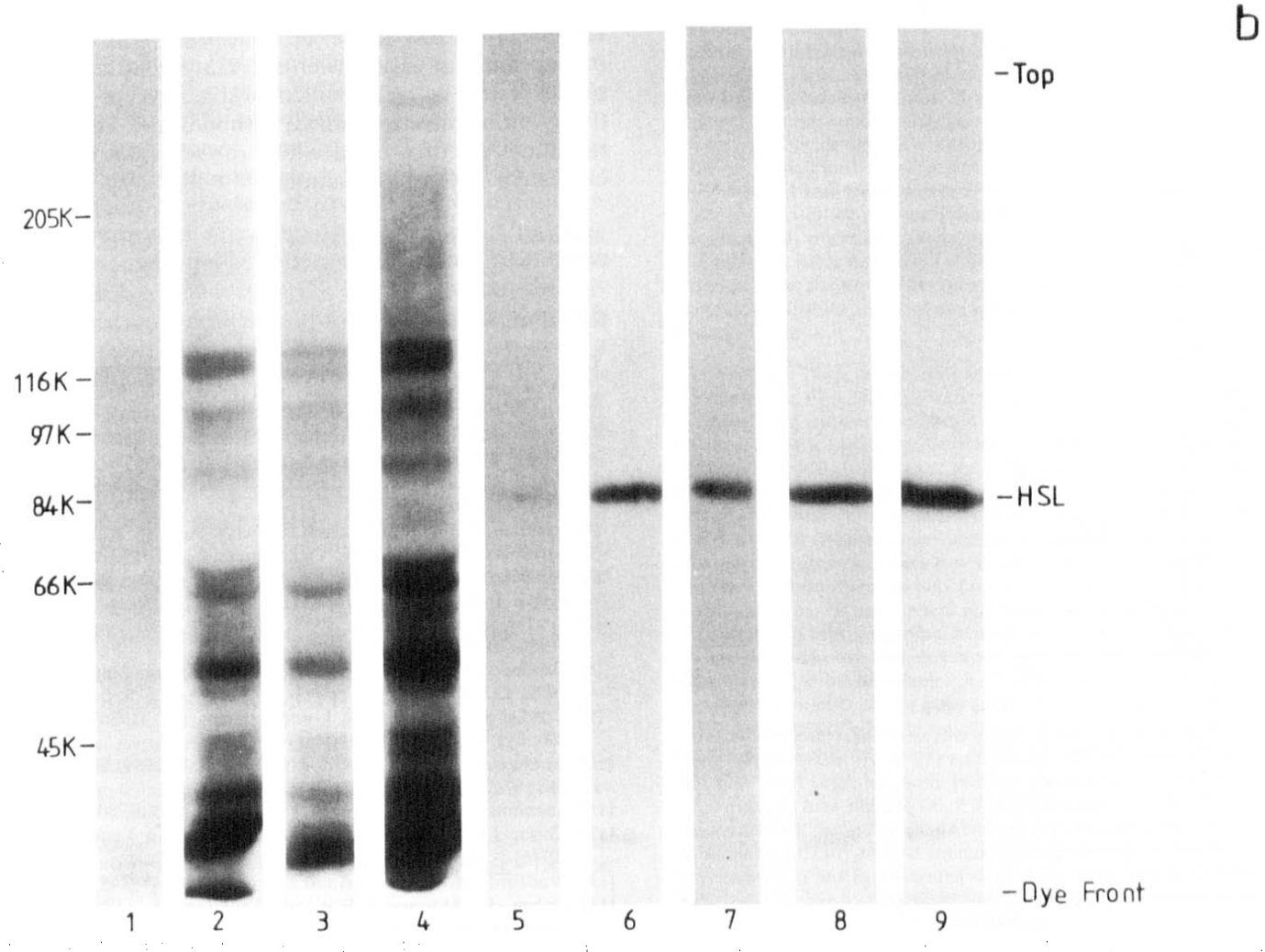
Fig. 1. Phosphorylation of HSL in intact macrophages. WEHI macrophages (a) or mouse peritoneal macrophages (b) were preincubated for 2 h at 37°C in the presence of 0.5 mCi/ml, 0.8 mM[^{32}P]orthophosphate and then incubated for a further 15 min in the presence of 1.5 mM dibutyryl cyclic AMP and 3-isobutyl-1-methylxanthine (lanes 2 and 6), 1 μM okadaic acid (lanes 3 and 7), or all three agents (lanes 4 and 8). Control incubations were carried out in the absence of these additions (lanes 1 and 5). Extracts were prepared as described in Materials and Methods and samples (approximately 75 μg protein) analysed by SDS-PAGE and autoradiography (lanes 1–4) or else incubated with anti-HSL immunoglobulin and the immunoprecipitates analysed by SDS-PAGE and autoradiography (lanes 5–8). Lane 9 contains a sample (0.25 μg) of purified HSL phosphorylated by cyclic AMP-dependent protein kinase using [γ-^{32}P]ATP.

soluble extract assayed for cholesterol ester hydrolase activity at pH 7.0 against cholesteryl [^{3}H]oleate in ethanolic suspension [12]. One unit of enzyme activity catalyses the release of 1 μmol fatty acid per min. Protein concentration was determined by the method of Bradford [13].

3. RESULTS AND DISCUSSION

We have presented evidence previously that the WEHI macrophage cell-line contains HSL which is responsible for the neutral cholesterol esterase activity in these cells [3]. A key feature of HSL is its regulation by phosphorylation by cyclic AMP-dependent protein kinase, but this has not been demonstrated within intact macrophages. To investigate whether this regulatory mechanism operates within macrophages, WEHI cells were preincubated in the presence of [^{32}P]orthophosphate, and possible phosphorylation of HSL investigated by incubation with various agents followed by immunoprecipitation of the polypeptide from cell extracts. As shown in Fig. 1a, incubation of the cells resulted in phosphorylation of the 84 kDa HSL polypeptide. Treatment of the cells with dibutyryl cyclic AMP, and 3-isobutyl-1-methylxanthine, an inhibitor of cyclic AMP phosphodiesterases, caused a significant increase in the phosphorylation state of HSL (the increase, as estimated by densitometric scanning of the autoradiograph, was approximately 8-fold), indicating that the phosphorylation was dependent on the intracellular concentration of cyclic AMP. Addition of okadaic acid, a potent inhibitor of protein phosphatases 1 and 2A [14] also caused a significant increase (approximately 7-fold) in the phosphorylation of HSL. The effects of agents acting via cyclic AMP and of okadaic acid were additive, the overall increase in phosphorylation being approximately 14-fold.

Cell-lines must be used with caution in that they do not necessarily possess all the essential characteristics of the cell type from which they are derived. In view of this, it was considered essential to show that HSL is also present in mouse peritoneal macrophages and that the enzyme can be regulated by phosphorylation in such cells. As shown in Fig. 1b, HSL is present in peritoneal macrophages and can be phosphorylated in these cells in a cyclic AMP-dependent manner.



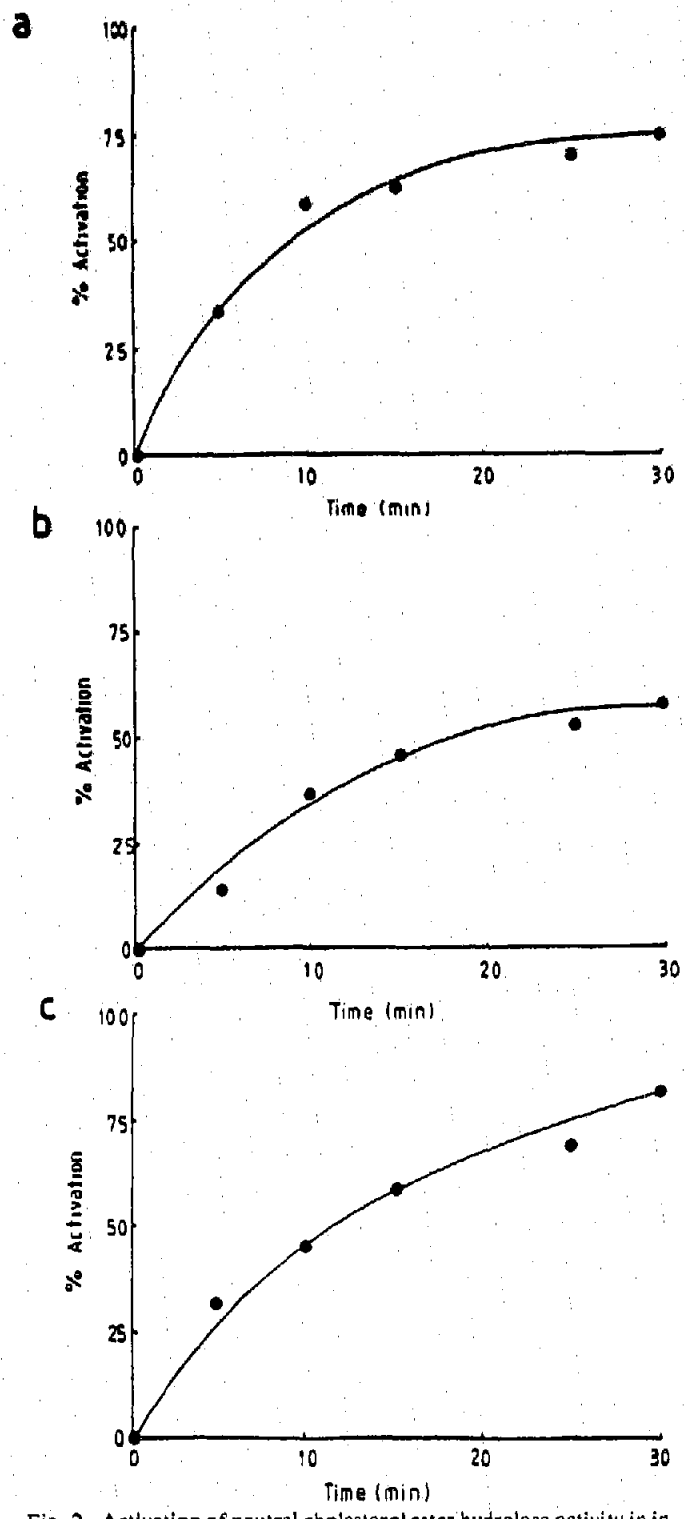


Fig. 2. Activation of neutral cholesterol ester hydrolase activity in intact macrophages. WEHI macrophages (2×10^6 cells/sample) were incubated at 37°C with either 1.5 mM dibutyryl cyclic AMP and 100 μM 3-isobutyl-1-methylxanthine (a), 1 μM okadaic acid (b), or a mixture of all three reagents (c) for the times indicated. Extracts were prepared and assayed for activity against cholesterol [^{3}H]oleate as in Materials and Methods. Activity is expressed as the percentage increase in neutral cholesterol ester hydrolase activity over the zero time control value.

Incubation of WEHI cells with dibutyryl cyclic AMP and 3-isobutyl-1-methylxanthine also caused a time-dependent increase in neutral cholesterol ester hydrolase activity, with maximum activation being approximately 75% above control values (Fig. 2a). These agents were shown previously to have a similar effect on cholesterol ester hydrolase in another macrophage cell-line, namely PD388$_1$, where activation was also cyclic AMP-dependent and did not result from other known effects of butyrate on cultured cells, such as induction of protein synthesis [8]. Treatment of intact WEHI cells with okadaic acid was also found to result in a time-dependent increase in neutral cholesterol ester hydrolase activity, with maximum activation being approximately 60% above control values (Fig. 2b). In these studies okadaic acid was used in the incubation medium at a final concentration of 1 μM, suggesting that both protein phosphatases 1 and 2A would be inhibited in the cell [14]. The finding that neutral cholesterol ester hydrolase activity is increased by okadaic acid is further strong evidence that phosphorylation plays a direct role in control of cholesterol ester metabolism in macrophages.

The hormonal regulation of cholesterol ester mobilisation in macrophages, via phosphorylation of HSL, may prove to be of therapeutic value in the management of atherosclerosis. Having established a model system whereby control of macrophage HSL activity can be directly studied, it should now be feasible to study whether extracellular agents can facilitate cholesterol ester mobilisation.

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