Expression of hormone-sensitive lipase mRNA in macrophages

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Abstract Macrophages contain a neutral cholesteryl ester hydrolase that can be activated by cAMP-dependent protein kinase. Immunological studies strongly suggest that hormone-sensitive lipase (HSL) is probably responsible for the cholesteryl ester hydrolase activity in macrophages; however, due to the very low level of expression in macrophages, it has been difficult to determine whether the macrophage cholesteryl ester hydrolase and adipose HSL are, in fact, products of the same gene. We have used the sensitive polymerase chain reaction (PCR) technique to demonstrate expression of HSL mRNA in resident and thioglycollate-elicited mouse peritoneal macrophages, as well as in the P388D$_1$ mouse macrophage cell line. PCR was performed using oligonucleotide primer sequences present on adjacent exons of the mouse HSL gene to allow discrimination between products derived from HSL mRNA or genomic DNA sequences; specificity of the PCR was demonstrated by the absence of a product in liver, which does not express HSL mRNA. Northern blot analysis of poly (A)$^+$ RNA from peritoneal macrophages with a mouse adipose HSL cDNA probe demonstrated a low abundance of mRNA of 3.2 kb, identical in size to HSL mRNA in adipose tissue. These findings, together with the results of previous studies demonstrating similarities between HSL and macrophage neutral cholesteryl ester hydrolase, strongly support the conclusion that both are products of a single gene. The development of a PCR assay for HSL mRNA may allow further study of the regulation of neutral cholesteryl ester hydrolase expression in macrophages and foam cells, and its potential role in atherogenesis.—**Khoo, J. C., K. Reue, D. Steinberg, and M. C. Schotz.** Expression of hormone-sensitive lipase mRNA in macrophages. *J. Lipid Res.* 1993. **34:** 1969–1974.

Supplementary key words cholesteryl ester hydrolase • mouse peritoneal macrophages • P388D$_1$ macrophage cell line • polymerase chain reaction

Cholesteryl ester-laden foam cells of macrophage origin are a prominent feature of fatty streaks, the earliest atherosclerotic lesion, and also play a role in progression of fatty streaks to more advanced, clinically significant lesions (1–5). Lesion regression has been demonstrated in experimental atherosclerosis (6–9), accompanied by a large decrease in arterial cholesteryl ester concentration and virtual disappearance of fatty streak lesions. Lesion regression has also been demonstrated in human atherosclerosis (10). As regression is induced by simply lowering plasma lipoprotein levels, it presumably reflects a decrease in rates of deposition of cholesterol as esters to a level below the rate of cholesteryl ester hydrolysis and mobilization. The enzyme presumably responsible for mobilization of stored cholesteryl esters is the neutral cholesteryl ester hydrolase (11, 12), thought to be identical to hormone-sensitive lipase (HSL) (12–14). Free cholesterol released by the action of cholesteryl ester hydrolase can either leave the cell, provided there is an adequate concentration of cholesterol acceptors available (e.g., high density lipoprotein), or be returned to the cholesteryl ester pool by the action of acyl-CoA:cholesterol acyltransferase (ACAT) (15). Thus, whether a foam cell will increase or decrease its stores of cholesteryl esters depends on the relative activities of neutral cholesteryl ester hydrolase and ACAT. A better understanding of how these two enzyme activities are controlled could help clarify the process of lesion formation and lesion regression.

We have shown previously that adipose tissue contains a very high level of neutral cholesteryl ester hydrolase activity that can be activated by cAMP-dependent protein kinase (16, 17). As most of the cholesterol stored in adipose tissue exists as free cholesterol, the functional significance of this enzymatic activity is not clear. However, recent studies using both immunoblotting (18) and Northern blot analysis (19) have clearly established that the HSL of adipose tissue is the enzyme that catalyzes the hydrolysis of both triacylglycerols and cholesteryl esters. HSL has also been shown to be expressed in adrenals, ovaries, testes, placenta, mammary tissue, skeletal muscle, and heart (18–22).

Abbreviations: cAMP, cyclic AMP; HSL, hormone-sensitive lipase; PCR, polymerase chain reaction; TEG, 25 mM Tris-HCl/1 mM EDTA/20% glycerol (pH 7.4); MPM, mouse peritoneal macrophages; SSC, saline-sodium citrate buffer; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; MOPS, 4-morpholinepropanesulfonic acid; ACAT, acyl-CoA:cholesterol acyltransferase.

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We have previously demonstrated the presence of a cAMP-dependent protein kinase-activatable neutral cholesteryl ester hydrolase in J774 and P388D_1 macrophage cell lines (23, 24). This finding was confirmed and extended by Small, Goodacre, and Yeaman (13) and Small et al. (14). They have shown that an antibody against HSL of adipose tissue completely inhibits the neutral cholesteryl ester hydrolase activity in both mouse peritoneal macrophages and the WEHI macrophage cell line. The antibody recognized a protein band with a molecular mass of 84 kDa, identical to that of HSL. Their data suggest, but do not prove, that the neutral cholesteryl ester hydrolase of macrophages and the HSL of adipose tissue are both products of the same gene, the gene for HSL. In this report, we demonstrate for the first time the expression of HSL mRNA in both mouse peritoneal macrophages and the P388D_1 macrophage cell line using polymerase chain reaction (PCR) and RNA blot analysis.

MATERIALS AND METHODS

Cells

Resident and elicited mouse peritoneal macrophages were harvested by lavage of the peritoneal cavity of female Swiss-Webster mice with phosphate-buffered saline (PBS). Elicited macrophages were obtained by intraperitoneal injection of 2 ml of thioglycollate medium (DIFCO) 3 days before harvest. The cells from peritoneal lavage were plated on 60-mm Petri dishes at a density of 1×10^7 cells per plate in RPMI medium (GIBCO) containing 10% fetal bovine serum, 50 μg/ml gentamycin sulfate, and 0.25 μg/ml fungizone. After overnight incubation, nonadherent cells were removed by washing the plate three times with ice-cold PBS. The adherent cells were harvested in 25 mM Tris-HCl containing 1 mM EDTA and 20% glycerol, pH 7.4 (TEG), followed by sonication for 30 sec at 4°C.

The murine P388D_1 macrophage cell line was purchased from American Type Culture Collection. Cells were grown to confluence in 100-mm Petri dishes in RPMI medium supplemented with 10% fetal bovine serum, 50 μg/ml gentamycin sulfate, and 0.25 μg/ml fungizone. The monolayers of cultured cells were harvested and sonicated in TEG buffer for 30 sec at 4°C.

Assay for neutral cholesteryl ester hydrolase activity

Neutral cholesteryl ester hydrolase activity was assayed as described by Goldberg and Khoo (24). The assay mixture contained 0.0625 mM cholesteryl [1-^{14}C]oleate, 50 mM phosphate buffer (pH 7), 2 mM EDTA, 3 mg/ml bovine serum albumin, and 50–100 μg protein of cell sonicate. After 30 min incubation at 30°C, the [^{14}C]oleic acid released was extracted with chloroform–methanol–benzene 1:2.4:2, followed by the addition of NaOH. The cholesteryl ester hydrolase activity was expressed as nmol of free fatty acid released per mg protein per hour.

RNA isolation

Peritoneal macrophages from Swiss-Webster mice, epididymal adipose tissue and liver from BALB/c mice, and P388D_1 cells were frozen immediately and stored at −70°C. Total RNA was prepared using guanidinium thiocyanate (25). Poly (A)$^+$ RNA was isolated from total RNA using the PolyATtract mRNA Isolation System IV (Promega).

Detection of HSL mRNA using polymerase chain reaction (PCR)

One μg of poly (A)$^+$ RNA from mouse adipose tissue, liver, peritoneal macrophages, and P388D_1 cells was used as template for cDNA synthesis by reverse transcriptase with oligo (dT) primers as described (26). One-tenth of the resulting cDNA (5 μl) was utilized for the PCR. Primer sequences used for PCR were based on sequences derived from characterization of the mouse adipose HSL gene (Z. Li and K. Reue, unpublished data) and mouse apolipoprotein (apo)A-IV cDNA (26). Primers used for PCR amplification of mouse HSL were as follows: forward primer, 5'-GCTGGTGCAGAGAGACAC-3' (nucleotides 1515–1532 of mouse HSL coding sequence); reverse primer, 5'-GAAAGCAGCGCGCACGCG-3' (nucleotides 1906–1923 of mouse HSL coding sequence). The HSL primers are located 408 nucleotides apart on the mouse HSL mRNA, but are located on separate exons of the mouse HSL gene, such that amplification of genomic DNA will include an intron of approximately 2,000 nucleotides in addition (see Fig. 1). Primers used for PCR amplification of mouse apoA-IV were as follows: forward primer, 5'-CCTCTTCCAGGACAAACTTG-3' (nucleotides 272–291 of apoA-IV cDNA); reverse primer, 5'-TTCTCAGTTGCTTTATTGA-3' (nucleotides 1421–1439 of apoA-IV cDNA). The apoA-IV primers amplify a fragment of 1148 nucleotides from mouse apoA-IV mRNA. Amplification reactions were carried out for 30 cycles with denaturation at 94°C for 1 min, annealing at 55°C for 45 sec, and extension at 72°C for 2 min (increased to 10 min for the final cycle). PCR products were analyzed on 3% agarose gels containing 3 parts NuSieve agarose and 1 part SeaKem agarose (FMC Bioproducts).

Cloning of HSL cDNA from mouse adipose RNA

One μg of poly (A)$^+$ RNA from mouse adipose tissue was used as a template for cDNA synthesis and amplified by PCR as above. The primers used were as follows: forward primer, 5'-AAACGCAACGAGACGGGC-3' (nucleotides 1201–1218 of the mouse HSL coding region); reverse primer, 5'-TTCCCAGCTCAGGTCAGCGGTGCAGCA (nucleotides 3003–3029 of the mouse HSL coding region). The resulting 1828 nucleotide product was

purified by electrophoresis and ligated into the *SmaI* site of the plasmid pGEM2 (Promega). This partial mouse HSL cDNA was used as a probe for Northern hybridization.

Northern hybridization

Poly (A)$^+$ RNA was electrophoresed through 1% agarose containing 1.1% formaldehyde and 1 × MOPS buffer (0.02 M 4-morpholinepropanesulfonic acid, 0.005 M sodium acetate, 0.001 M EDTA, pH 7), and then transferred to Hybond-N+ positively charged nylon membrane (Amersham) by vacuum blotting (27). RNA was fixed to membranes by UV-crosslinking and hybridized to mouse HSL cDNA that had been radiolabeled with [^{32}P]dCTP using a Random Primed DNA Labeling Kit (Boehringer Mannheim). Hybridization was performed with 2 × 10^6 cpm probe/ml in 0.5 M sodium phosphate (pH 7.0), 1 mM EDTA, 1% sodium dodecyl sulfate (SDS), and 1% bovine serum albumin at 65°C for 16 h. Sequential 30-min washes were performed at 65°C in 2× saline-sodium citrate buffer (SSC)/0.1% SDS, 1 × SSC/0.1% SDS, 0.5 × SSC/0.1% SDS, and 0.1 × SSC/0.1% SDS. Membranes were exposed to Kodak XAR film with intensifying screens at −70°C.

RESULTS

Neutral cholesteryl ester hydrolase activity in mouse tissues

The levels of neutral cholesteryl ester hydrolase activity were determined in various mouse tissues (adipose tissue, adrenal, and heart), and in resident and thioglycollate-elicited peritoneal macrophages. As shown in **Table 1,** adipose tissue contained the highest level of neutral cholesteryl ester hydrolase activity, approximately 3-fold higher than that of adrenal and 15-fold higher than that of heart. Neutral cholesteryl ester hydrolase activity in both resident and thioglycollate-elicited macrophages was extremely low, only 3–5% that in adipose tissue. To eliminate the possibility that the activity detected in peritoneal macrophages was due to contamination with omental adipocytes or pre-adipocytes, monolayers of macrophages were washed extensively to remove non-adherent cells prior to harvesting for enzymatic assay and preparation of poly (A)$^+$ RNA (see Methods). In addition, the experiment was also carried out with P388D$_1$ cells, an established mouse macrophage cell line that has been used previously to study the metabolism of acetylated low density lipoprotein (28) and hypertriglyceridemic very low density lipoprotein (29). Neutral cholesteryl ester hydrolase activity was present in P388D$_1$ macrophages at approximately 85% the level detected in resident peritoneal macrophages (Table 1). With this established cell line, the problem of adipocyte contamination was completely eliminated.

TABLE 1. Comparison of neutral cholesteryl ester hydrolase activity in mouse adipose tissue, adrenal, heart, and macrophages

Tissue or Cell Type	Neutral Cholesteryl Ester Hydrolase Activity
	nmol FFA/mg protein/h
Adipose tissue	174.5 ± 6.0
Adrenal	48.8 ± 1.3
Heart	12.1 ± 0.6
Resident MPM	5.13 ± 0.73
Thioglycollate-elicited MPM	8.68 ± 0.92
P388D$_1$ cells	4.32 ± 0.36

Tissues or packed cells were homogenized in three volumes of TEG buffer and centrifuged at 10,000 rpm for 20 min in microfuge. The supernatant fractions were assayed for neutral cholesteryl ester hydrolase activity as described in Materials and Methods; MPM, mouse peritoneal macrophages. Each point represents the mean of triplicate determinations ± SD.

PCR detection of mRNA for HSL in macrophages

To determine whether the neutral cholesteryl ester hydrolase activity in macrophages and the HSL activity of adipose tissue might be products of the same gene, we searched for evidence of HSL mRNA in mouse macrophages. Our initial attempts using Northern hybridization to total and poly (A)$^+$ RNA (up to 8 μg) yielded negative results, consistent with the very low level of neutral cholesteryl ester hydrolase activity observed in macrophages (Table 1). We therefore turned to the more sensitive PCR technique. RNA from mouse adipose tissue, liver, peritoneal macrophages, and P388D$_1$ cells was converted to cDNA using reverse transcriptase and an oligo (dT) primer. The resulting cDNA was amplified with primers specific for mouse adipose tissue HSL (see Methods). The primers chosen for PCR are located on adjacent exons of the mouse HSL gene such that PCR products generated from reverse transcribed HSL mRNA (408 nucleotide product) can be distinguished from products generated from genomic DNA contamination (approximately 2,400 base nucleotide product) (**Fig. 1**).

As shown in **Fig. 2** (left), the expected HSL-specific 408 nucleotide PCR product was generated from RNA prepared from mouse adipose tissue (lanes 3, 4), peritoneal macrophages (lanes 5, 6), and P388D$_1$ cells (lanes 7, 8); in contrast, no HSL amplification product was produced from liver RNA (lanes 1, 2). To demonstrate that RNA in the liver samples was intact, PCR was performed using primers specific for mouse apoA-IV, which is expressed in liver, but not in adipose tissue or macrophages (Fig. 2, right). Thus, amplification with apoA-IV primers produced an 1148 nucleotide product from liver; adipose tissue and macrophage RNA were negative. These results indicate that HSL mRNA is expressed in mouse adipose tissue, peritoneal macrophages, and P388D$_1$ cells, but not in liver.

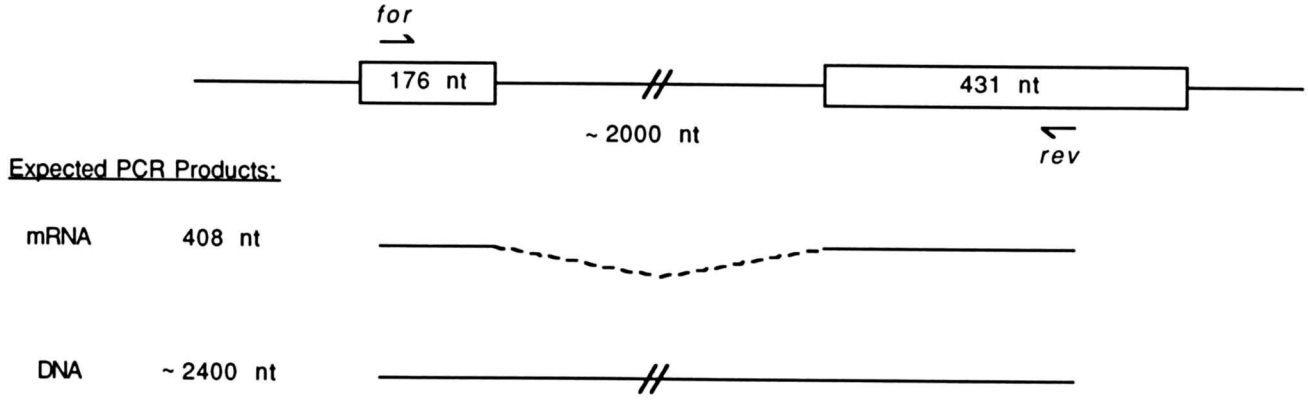

Fig. 1. Strategy to detect HSL mRNA by PCR. Position of oligonucleotide primers (arrows) are shown in relation to mouse HSL gene structure (Z. Li and K. Reue, unpublished data). The forward primer (for) is located on exon 7, and the reverse primer (rev) on exon 8. The 2000 nucleotide intervening intron is also shown. The positioning of primers on separate exons allows distinction between PCR products generated from reverse transcribed mRNA (408 nucleotide product) and from genomic DNA (2400 nucleotide product).

To confirm that the PCR products obtained from macrophages contained HSL sequences, restriction enzyme digestion was performed. The 408 nucleotide PCR products from adipose tissue and from macrophages were digested with the enzymes *Hinc*II and *Ava*I. The restriction pattern observed was that expected from the mouse adipose HSL sequence, with fragments of 259, 96, and 53 nucleotides (data not shown).

Northern blot analysis of mRNA for HSL in macrophages

Although the PCR results described above provide evidence for the expression of HSL mRNA in peritoneal macrophages, they do not provide information concerning size of the mRNA or the relative abundance in macrophages compared to that of adipose tissue. To address these issues, we performed Northern hybridization using a large quantity (25 μg) of poly (A)$^+$ RNA prepared from peritoneal macrophages. As shown in **Fig. 3,** a mouse adipose HSL cDNA probe hybridized to an mRNA of approximately 3.2 kb in both adipose tissue and macrophages. A 3.2-kb mRNA was also detected in RNA from P388D$_1$ cells (not shown). It is evident from the relative intensities of hybridizing bands that HSL mRNA is expressed in peritoneal macrophages at a very low level compared to that in adipose tissue, which may explain why it has not previously been detected by Northern blot analysis. As reported by Holm et al. (19), the expression of HSL mRNA in rat heart and skeletal muscle also was low and became detectable only when poly (A)$^+$ RNA was used.

DISCUSSION

The findings reported here clearly establish that HSL mRNA is expressed in mouse peritoneal macrophages

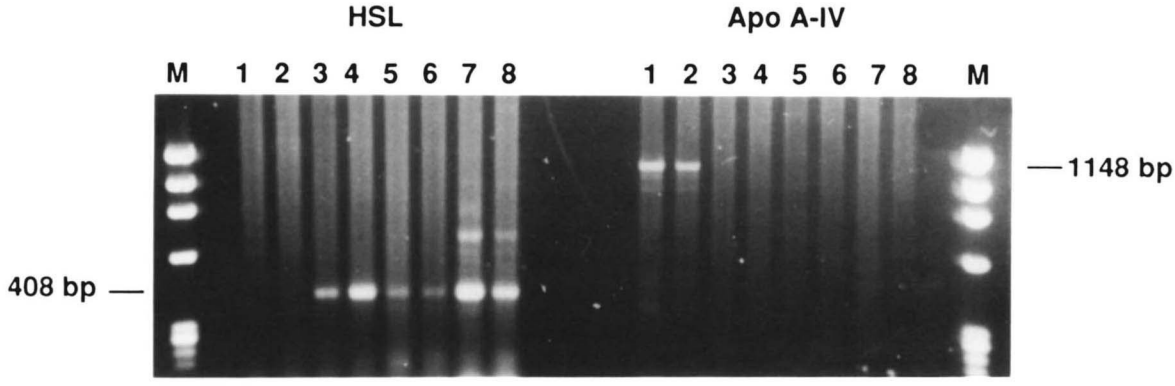

Fig. 2. Detection of HSL mRNA in macrophages by PCR. RNA from mouse liver, adipose tissue, peritoneal macrophages, and P388D$_1$ cells was reverse transcribed and amplified by the PCR with primers specific for HSL (left) or apoA-IV (right). The expected 408 nucleotide product of HSL and 1148 nucleotide product of apoA-IV are indicated by arrows. Lanes 1 and 2, liver; lanes 3 and 4, adipose tissue; lanes 5 (resident) and 6 (thioglycollate-elicited), peritoneal macrophages; and lanes 7 and 8, P388D$_1$ cells.

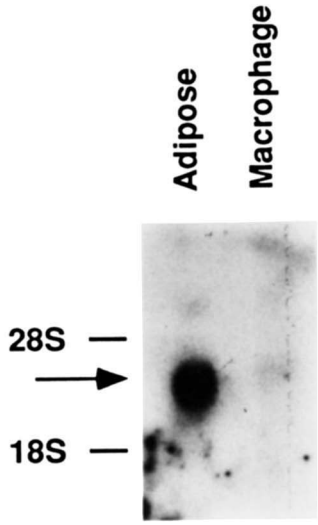

Fig. 3. Northern hybridization of HSL mRNA in macrophages. Poly $(A)^+$ RNA from adipose tissue (2 μg) and peritoneal macrophages (25 μg) were electrophoresed, blotted to nylon, and hybridized with a radiolabeled mouse HSL cDNA probe. Hybridizing bands of 3.2 kb are indicated by arrow. The blot was exposed to film for 5 days at −70°C.

and in the $P388D_1$ macrophage cell line. The level of expression is extremely low, consonant with the much lower levels of the enzymatic activity in macrophages compared to adipose tissue (Table 1). Nevertheless, it was possible to demonstrate the presence of HSL mRNA using Northern blot analysis when a large amount of poly $(A)^+$ RNA was used. The use of PCR allowed an unambiguous demonstration that the HSL gene is expressed in these macrophages. Because of the extreme sensitivity of the method, special care was taken to rule out the presence of contaminating adipocytes in the macrophage preparations. The similar results with the cultured $P388D_1$ cells avoids that problem altogether. Finally, the use of primers separated by an intron eliminated the problem of potential contamination with genomic DNA.

The fact that the HSL gene is expressed in macrophages does not necessarily establish that it is responsible for all or even a part of the neutral cholesteryl ester hydrolase activity of these cells. However, a number of facts add strength to the proposition that it is the dominant neutral cholesteryl ester hydrolase, regulating the rate of mobilization of stored cholesteryl esters. First, antibodies raised against HSL from adipose tissue completely inhibited neutral cholesteryl ester hydrolase activity in macrophages, and those antibodies recognize a protein band with an apparent molecular weight equal to that of HSL (13). Second, the properties of the neutral cholesteryl ester hydrolase in macrophages and its regulation by cAMP-dependent protein kinase are similar to or identical with the properties of HSL in other tissues (14, 16, 17). Third, stimulation of neutral cholesteryl ester hydrolase by cAMP analogs enhances the rate of mobilization of cholesteryl esters from J774 macrophages (30). Thus, the case is strong that HSL is the dominant neutral cholesteryl ester hydrolase in macrophages, but definitive proof may require experiments using antisense RNA or gene "knockout" experiments. Now that the mouse HSL gene has been cloned (Z. Li and K. Reue, unpublished data), such experiments are possible.

HSL has been identified in a large number of tissues. Some of the foam cells in developing atherosclerotic lesions are derived from smooth muscle cells (31). Hajjar and coworkers have demonstrated neutral cholesteryl ester hydrolase activity in arterial smooth muscle cells that can be activated by cAMP-dependent protein kinase (32). Obviously, it will be of interest to determine whether this activity is also attributable to the HSL gene.

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