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Mechanism of hormone-stimulated lipolysis in adipocytes: Translocation of hormone-sensitive lipase to the lipid storage droplet

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ABSTRACT **Hormone-sensitive lipase activity (HSL), which is found in the supernatant of centrifuged homogenates of lipolytically quiet isolated rat adipocytes, was greatly reduced in or absent from the supernatant of lipolytically stimulated cells. The lipase was purified 100- to 250-fold from the supernatant of lipolytically quiet cells to 10–20% purity by a single passage over phenyl-Sepharose resin with high (>70%) activity yields. Western blotting of adipocyte homogenate fractions with polyclonal antiserum raised against HSL showed that the enzyme shifted quantitatively from the supernatant of control cells to the floating "fat cake" of lipolytically stimulated cells. A similar shift to the fat cake was observed when cells were disrupted by hypotonic lysis and centrifugation rather than by homogenization. We propose that upon lipolytic activation of adipocytes and phosphorylation of HSL by cAMP-dependent protein kinase, the critical event is not an increase in catalytic activity (i.e., turnover number) but a translocation of the lipase to its substrate at the surface of the lipid storage droplet.**

Food energy in animals is stored primarily as triacylglycerols in lipid storage droplets of adipocytes. Lipolysis, the process that liberates fatty acids and glycerol from stored lipid, is activated by hormones that stimulate cAMP formation and subsequent phosphorylation of the rate-limiting enzyme of lipolysis, hormone-sensitive lipase (HSL) (for review, see refs. 1 and 2). The enzyme has been purified to near homogeneity (3) and the gene has been cloned from a rat cDNA library (4). Despite such advances, a remaining enigma is the great disparity between the lipolytic response in adipocytes upon increase of cellular cAMP, usually >50-fold, and the meager stimulation of HSL activity upon phosphorylation of the enzyme *in vitro* with cAMP-dependent protein kinase (A-kinase), generally <2-fold (1, 2, 5). Such findings suggest that an increase in HSL catalytic activity (i.e., turnover number) upon phosphorylation falls short of explaining the biological action of lipolytic hormones.

A possible explanation for this disparity lies in the observations of Hirsch and Rosen (6), who found that lipolytic stimulation led to a loss of HSL activity from the so-called infranate fraction, the aqueous fraction below the floating fat cake, of homogenates of cultured 3T3-L1 adipocytes and a gain of activity in the particulate fraction. Thus, redistribution of the enzyme may be an important consequence of stimulation and phosphorylation. Although Hirsch and Rosen (6) did not examine the lipid fraction or fat cake of the homogenates of the cultured cells, in previous studies with ^{32}P-loaded rat adipocytes we tentatively located HSL primarily, if not exclusively, in the fat cake from stimulated cells (ref. 7, figure 1; ref. 8, figure 2). Although we had no means of locating the enzyme in unstimulated cells, the supernatant of unstimulated adipocytes has served as the primary source for HSL purification (1–3). The present paper demonstrates that HSL translocates from one compartment to another upon elevation of A-kinase activity. A preliminary account of this work has appeared (9).

EXPERIMENTAL PROCEDURES

Materials. Sprague–Dawley rats were purchased from Charles River Breeding Laboratories or Taconic Farms, phenyl-Sepharose CL-4B from Sigma, and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) from Fluka. All other materials were from sources previously identified (7, 8).

Cell Preparation, Incubations, and Subcellular Fractionation. Adipocytes were isolated from the epididymal fat pads of 180- to 200-g rats according to the method of Rodbell (10), and incubations were carried out in Krebs–Ringer buffer, pH 7.4, supplemented with 30 mM Hepes. Adenosine (200 nM) was included in all media to suppress cAMP production during cell isolation (11).

Purification of HSL from Rat Adipocytes. HSL was purified from the postmicrosomal (160,000 × g) supernatant fraction of homogenates (12), which was adjusted to 10 mM NaF, acidified to pH 5.2 (13) with acetic acid in a stirring ice bath, equilibrated at pH 5.2 for 15 min, and centrifuged at 20,000 × g for 45 min. Typically, the acid precipitate contained 100% of the starting HSL activity and 25% of the initial protein, representing primarily removal of contaminating exogenous bovine serum albumin (BSA). A resuspension medium containing 20 mM sodium phosphate buffer (pH 7 at 4°C), 1 mM EDTA, 0.1 mM benzamidine, leupeptin (5 μg/ml), and 0.2% CHAPS was added (≈100 μl) to the acid precipitate derived from each rat. Thorough solubilization of this precipitate was essential for maximum yield of HSL. The precipitate was suspended by vigorous and repeated aspiration and ejection with an automatic pipettor, until the opaque solution contained minimal visible particulate material, and homogenized with 10 strokes in a tight-fitting ground-glass homogenizer. The volume was adjusted to 125 μl for each rat used in the preparation, and the solution was centrifuged at 40,000 × g

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Abbreviations: HSL, hormone-sensitive lipase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; A-kinase, cAMP-dependent protein kinase; PIA, (R)-(−)-N^6-(1-methyl-2-phenylethyl)adenosine; BSA, bovine serum albumin.

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for 25 min to remove remaining particulate matter, usually minimal by this point. Nearly all of the HSL activity was recovered in the clear supernatant from this centrifugation. Magnesium acetate (2 mM) was added and the material was centrifuged at 120,000 × *g* for 60 min in a Ti SW60 rotor (Beckman), yielding a gelatinous pellet of 200- to 220-kDa protein(s), which was discarded.

Phenyl-Sepharose resin was washed three times with 2 volumes of eluting solution that contained 25 mM Tris·HCl (pH 7), 1 mM EDTA, 0.1 mM benzamidine, leupeptin (5 μg/ml), and 30% (vol/vol) glycerol. The above 120,000 × *g* supernatant was introduced into the resin (0.5 ml per ml of resin) and mixed by gentle rocking overnight at 4°C. Batch loading was necessary for successful resolution of HSL from other proteins during phenyl-Sepharose chromatography. The slurry was poured into a column, typically 5.3 cm in diameter and 3 cm high for a preparation derived from 100 rats, and washed with 10 column volumes of eluting solution followed by 10 volumes of eluting solution containing 20% ethylene glycol. HSL was eluted with a linear gradient of eluting solution containing 20–70% ethylene glycol. The total elution volume was 3 ml for each rat used in the preparation; the rate of change of ethylene glycol concentration was 0.16%/ml at an elution rate of 15 ml/hr, and 2.5-ml fractions were collected. Peak activity fractions were pooled and diluted 3.5-fold with the eluting solution containing 0.05% CHAPS and concentrated in a 200-ml Amicon chamber with a PM30 membrane. The enzyme was concentrated further in a 10-ml chamber with the same filter until the final solution was approximately 30 times more concentrated than the initial pooled column elution.‖ Typically, for a preparation from 100 rats, the enzyme solution was reduced to 2.5–3 ml, aliquoted, and stored at −70°C. The enzyme was stable indefinitely under these conditions.

Assay for HSL Activity. The assay mixture (350 μl) contained 177 μM [^{14}C]cholesteryl oleate as substrate (45,000 cpm), 44 mM sodium phosphate (pH 7.0), and 2.45% defatted BSA. After incubation at 37°C for 30 min, the reaction was stopped by addition of 50 μl of 2 M NaOH to give a final pH of 12. The hydrolyzed sodium oleate was extracted by addition of 1.5 ml of methanol/chloroform/benzene (2.4:2:1, vol/vol) with 100 μM oleic acid as carrier (14). Following vigorous mixing for 15 sec, the emulsion was centrifuged at 2200 rpm (maximum radius, 15 cm) in a clinical IEC centrifuge for 10 min, and an aliquot of the upper, aqueous phase was withdrawn for liquid scintillation counting. At all stages of purification care was taken to assess linearity of enzyme activity with respect to protein concentration by testing serial dilutions of samples.

Preparation of Antisera Against HSL and Western Blotting. Antisera against partially purified HSL were produced by injecting rabbits with protein that had been transferred to nitrocellulose after SDS/PAGE (8). For Western blotting, proteins were separated by SDS/PAGE, transferred to nitrocellulose, and blocked with 4% dry milk in 50 mM Tris·HCl, pH 7.5/160 mM NaCl/0.05% Nonidet P-40. Strips were incubated with a 1:1000–1:2000 dilution of antiserum against HSL. Immunoreactivity was visualized with alkaline phosphatase-linked goat anti-rabbit IgG and the 5-bromo-4-chloro-3-indolyl phosphate (*p*-toluidine salt)/*p*-nitro blue tetrazolium chloride staining procedure (Bio-Rad).

Subcellular Localization of HSL. Adipocytes were washed in BSA-free Krebs–Ringer/Hepes medium, suspended at a cell concentration of ≈20% (vol/vol), and incubated briefly with lipolytic effectors as indicated in figure legends, after which cells were permitted to rise and the infranate was withdrawn. Each milliliter of floating cell mass, equivalent to 0.5–0.75 ml of cells packed by centrifugation, was homogenized in a glass homogenizer with 1 ml of a medium containing 254 mM sucrose, 25 mM Tris·HCl (pH 7.4), 1 mM EDTA, 0.1 mM benzamidine, 20 μM leupeptin, 20 mM NaF, and soybean trypsin inhibitor (2 mg/ml). After centrifugation at 35,000 × *g*, the membrane pellets and fat cakes were suspended in Laemmli sample buffer containing 1% SDS, and all fractions were frozen. For SDS/PAGE, equivalent samples, typically containing a portion of each fraction representing 1/100th of that derived from a single rat, were added to Laemmli sample buffer containing 20% SDS. The high SDS concentration, typically 50 μl of 20% SDS per 10 μl of fat cake, was necessary in order to provide sufficient detergent to dissolve the lipid in the sample.

Alternatively, cells were lysed by rapid centrifugation in a hypotonic medium. Aliquots of cells incubated with lipolytic effectors were added to the above homogenizing medium without sucrose and centrifuged immediately at 35,000 × *g* for 15 min. Thereafter, subcellular fractions were processed as described for the homogenized cells.

RESULTS

Purification of HSL. To determine the subcellular location of HSL under various conditions of cell stimulation, it was necessary to obtain sufficient enzyme for antibody production. Thus, one goal of this study was to develop a procedure to purify HSL rapidly and with a high yield. The starting material for the purification was the supernatant derived from isolated adipocytes that were lipolytically unstimulated at the time of homogenization. Since it has been reported that a detergent is necessary for manipulating HSL in solution (2), we included CHAPS during purification, but only in the medium for solubilizing the acid precipitate. In preliminary studies, we found that CHAPS, at up to 0.5%, neither inhibited activity nor rendered the enzyme labile. Although all experiments presented in this paper were performed with CHAPS, in other studies we found that elimination of CHAPS reduced the yield by 50% or less.

The key feature in the purification scheme was chromatography over phenyl-Sepharose and elution with a gradient of ethylene glycol (Fig. 1). The vast majority of endogenous proteins (>95%) as well as any remaining BSA were removed following washing of phenyl-Sepharose with 20% ethylene glycol. Since HSL represents the only known significant hydrolytic activity against cholesterol esters in adipocytes (2), hydrolysis of [^{14}C]cholesteryl oleate was used to monitor activity. Hydrolytic activity against [^{14}C]cholesteryl oleate was eluted at 45–65% ethylene glycol, and this single chromatographic step routinely effected a purification of from 100- to 250-fold as assessed by activity measurements. Yields of HSL activity from the phenyl-Sepharose column routinely were >70% of initial activity in the solubilized acid precipitate. A protein of 84 kDa, evident in silver-stained SDS/polyacrylamide gels, that was coeluted with hydrolytic activity (Fig. 2*B*) was identified as HSL. A-kinase-dependent phosphorylation of an 84-kDa polypeptide (Fig. 2*C*) corresponded precisely with cholesterol esterase activity and with the silver-staining protein of the same apparent molecular size (Fig. 2 *A* and *B*). Although both the nonretained material and early (<20%) ethylene glycol fractions contained proteins of ≈84 kDa, none of these fractions contained lipase activity. Moreover, the relative mass of the 84-kDa protein that appeared with cholesterol esterase activity was ≈0.05%

‖We found the quality of CHAPS to vary among suppliers. The product from Fluka could be concentrated considerably and the concentrate remained a clear solution, whereas the solutions of products from other suppliers became cloudy because they contained substantial amounts of an insoluble material that sedimented upon centrifugation. This material interfered with most subsequent purification procedures.

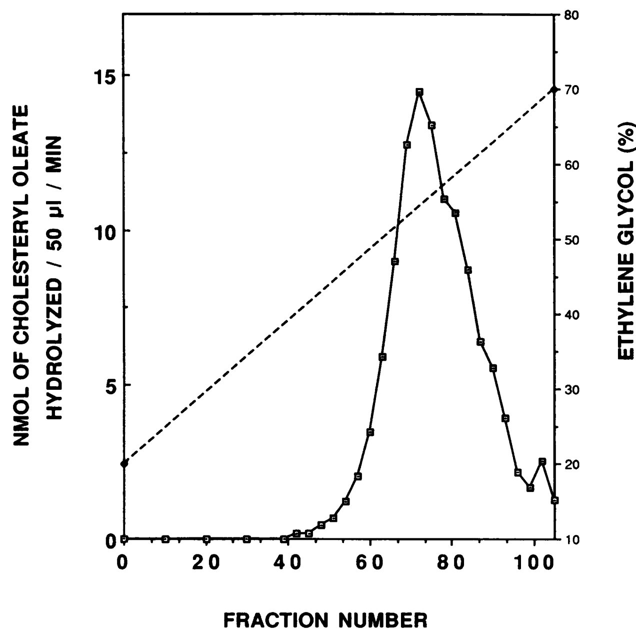

FIG. 1. Elution of HSL from phenyl-Sepharose with ethylene glycol. HSL was batch-loaded on phenyl-Sepharose as described under *Experimental Procedures* and was eluted with the indicated gradient of ethylene glycol.

of the starting material, in line with previous estimates of HSL abundance (3). Since the activity in the initial starting supernatant was preserved upon precipitation at pH 5.2 when stored at −80°C and activity in the phenyl-Sepharose elution was inhibited by diisopropyl fluorophosphate, known properties of HSL (2), it was concluded that 84-kDa molecule identified was indeed HSL.

It was possible to harvest a majority of the HSL at a relative purity of 10–20% (see legend to Fig. 2) by one chromatographic step, typically within 1 day. Although not necessary prior to phenyl-Sepharose chromatography, centrifugation in the Mg^{2+}-containing medium sedimented a gelatinous pellet containing substantial amounts of protein(s) of 200–220 kDa which appeared to form aggregates that interfered with a variety of concentrative and chromatographic procedures (data not shown).

Lipase activity in the peak fractions from the phenyl-Sepharose column declined slowly over several days at 4°C but was stable when concentrated and stored at −70°C. Pooled peak fractions were subjected to SDS/PAGE to isolate HSL for antibody production.

Localization of HSL in Subcellular Fractions. Although the supernatant of lipolytically quiescent adipocytes contained abundant cholesterol esterase/HSL activity, we found little or no activity in the supernatants of lipolytically stimulated cells, nor was there was any 84-kDa protein detectable by silver staining following phenyl-Sepharose chromatography (data not shown). Nearly all of the HSL was found in the supernatant fraction of unstimulated cells by Western blotting of gross adipocyte subcellular fractions with antiserum directed against HSL (Fig. 3), but little or no HSL was in the fat cake. By contrast, upon stimulation of cells with isoproterenol nearly all of the HSL was associated with the fat cake, accounting for the corresponding loss of HSL from the supernatant. No HSL was detected in the membrane fractions from either unstimulated or stimulated cells when cells were disrupted by homogenization.

One difficulty in interpreting the data in Fig. 3 is that the cellular subfractions were derived from mechanically homogenized cells, raising the possibility that dispersed lipids might

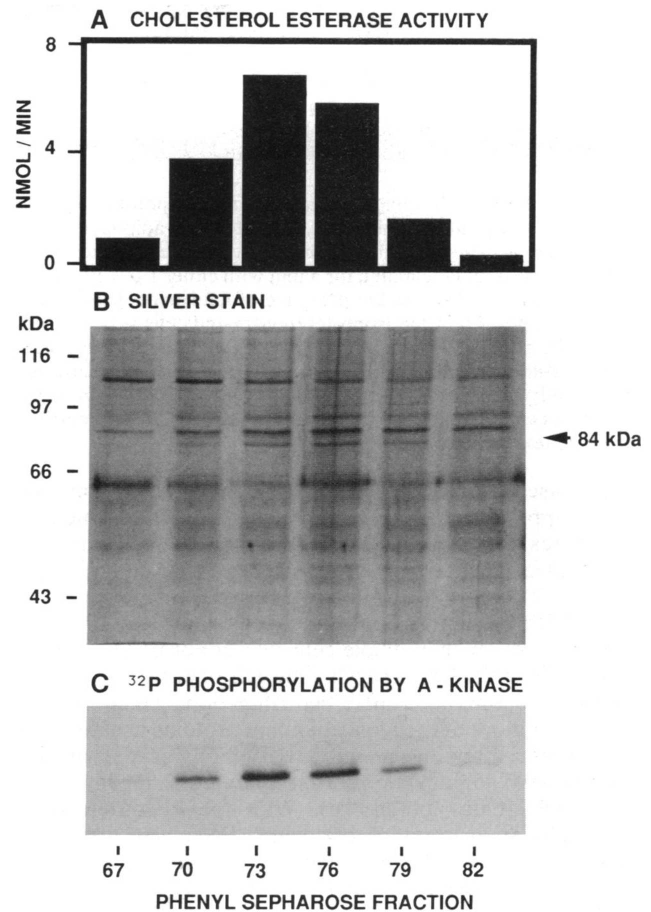

FIG. 2. Characterization of material in the peak activity fractions of the phenyl-Sepharose chromatography. (*A*) Cholesterol esterase activity. (*B*) Silver staining after SDS/PAGE. The dark material in the 60-kDa region is a silver-staining artifact that appeared also in lanes that were not loaded with protein. (*C*) *In vitro* phosphorylation by A-kinase and [γ-^{32}P]ATP. Phosphorylation conditions were as described in ref. 7.

have introduced artifacts, such as stripping of the lipase from particulate material, especially from the lipid droplet surface

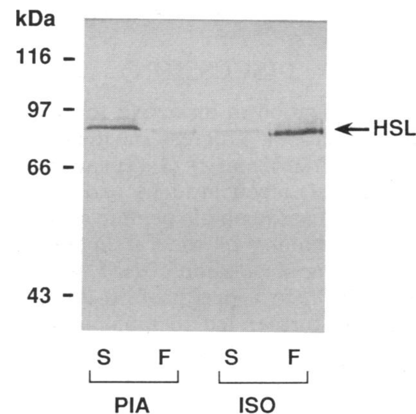

FIG. 3. Western blotting of adipocyte fractions with antiserum against HSL following homogenization of cells. Isolated rat adipocytes were washed and suspended in the Krebs–Ringer/Hepes solution in the absence of BSA and incubated for 5 min with either 1 μM (*R*)-(−)-N^6-(1-methyl-2-phenylethyl)adenosine (PIA) or 10 μM isoproterenol (ISO) in the presence of adenosine deaminase (1 unit/ml). The samples were homogenized and processed as described under *Experimental Procedures*. S, supernatant, also known as infranate under the fat cake; F, extract of the floating fat cake.

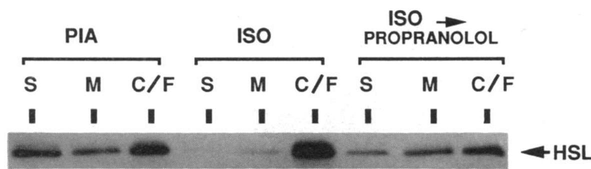

FIG. 4. Western blotting of fractions from hypotonically lysed cells with antiserum against HSL. Isolated rat adipocytes were washed and suspended in the Krebs–Ringer/Hepes solution in the absence of BSA and incubated for 5 min with either 1 μM PIA or 100 nM isoproterenol (ISO) in the presence of adenosine deaminase (1 unit/ml). A portion of the isoproterenol-treated cells was subjected to 10 μM propranolol for an additional 10 min. Homogenizing solution without sucrose was added and the cells were centrifuged immediately. S, supernatant; M, membranes; C/F, combination of unbroken cells and fat cake that floated at the top of the centrifuge tube (see text).

in the case of unstimulated cells. For this reason, an alternative approach was taken to disrupt cells in which the adipocytes were added to hypotonic medium and centrifuged immediately. This procedure yielded membrane and supernatant fractions, but the floating material in this case contained both the lipid from broken cells and many unbroken cells. The presence of whole cells was confirmed by viewing silver-stained SDS/polyacrylamide gels of proteins extracted from the floating layer after centrifugation, which revealed that half or more of the most abundant proteins usually found in the supernatant and membrane fractions of mechanically homogenized adipocytes were associated with the fat cake of lysed cells (data not shown). With the hypotonic lysis/centrifugation method of breakage, HSL was distributed approximately equally between the supernatant and membranes in unstimulated cells but disappeared from both fractions upon stimulation with isoproterenol. The loss of enzyme from the supernatant and membranes was accounted for by the gain in the floating material. Since, as noted above, many intact cells were trapped in the fat cake, considerable HSL was associated with the lipid in unstimulated cells, but the increase in lipase upon stimulation was clearly seen. In separate studies, we determined that addition of the β-adrenergic blocking agent propanolol reversed the phosphorylation of A-kinase phosphorylated proteins within 1–2 min under these incubation conditions. Propanolol caused the lipase to be redistributed to the supernatant and membranes, with a corresponding loss in the floating material (Fig. 4).

DISCUSSION

Resistance to purification in an active form was for many years a limiting factor in progress on the biochemistry of HSL. Ultimately, Fredrikson *et al.* (3) succeeded in developing a method that, albeit arduous and time-consuming, provided sufficient mass to obtain peptide sequence data that led eventually to cloning of the rat lipase. The present procedure permits rapid isolation of HSL with high activity yield, usually >70%, to a purity of 10–20%. Attempts to purify the enzyme further led to rapid losses in activity. However, after phenyl-Sepharose chromatography, the lipase is easily separated from other proteins by SDS/PAGE or by high-performance liquid chromatography over a Spherogel-TSK column (Altex 3000SW; 7.5-mm internal diameter × 30 cm) (unpublished work), providing a convenient method for examining remaining questions on the regulation of endogenous phosphorylation. HSL is reported to undergo phosphorylation at a "basal" site in addition to the A-kinase "regulatory" site (15). With the bovine enzyme, Ser-563 is the A-kinase site, whereas Ser-565 is a target for both calcium/calmodulin-dependent and AMP-activated protein kinases (16, 17). Interestingly, phosphorylation at these two sites is mutually exclusive. If Ser-565 were the basal site, this would provide an interesting and novel regulatory system in which activation of a phosphatase to remove the phosphate at Ser-565 would be a prerequisite for activation by A-kinase. Whether or not the basal site is phosphorylated in unstimulated cells is controversial. Although we found no radiophosphate in HSL isolated from unstimulated cells, others have (5, 15), and different cell incubation conditions may account for such differences. The availability of a method for rapidly harvesting lipase from cells might help to resolve this issue.

Despite its role as the gatekeeper of the vast majority of stored energy reserves, surprisingly little is known of the subcellular location of HSL in primary adipocytes, and as reviewed by Hirsch and Rosen (6) a confusing picture is presented by localization studies based solely on activity measurements. Examination by Western blotting in the present study reveals that the enzyme is in the aqueous supernatant phase of homogenates from lipolytically unstimulated adipocytes but is bound to the fat cake following lipolytic stimulation of cells. The data suggest a translocation of HSL from the cytosol to the lipid droplet upon lipolytic stimulation. Even with extremely mild disruption of cells by the hypotonic lysis/centrifugation method rather than homogenization, lipase distributes between the aqueous phase and pelleted membranes in unstimulated cells and, again, moves to the fat cake upon stimulation, a process that is reversed upon cessation of stimulation. Given the gentleness of cell disruption by this method and the likelihood that the pelleted material is composed primarily of cell ghosts with trapped cytosolic material, one presumes that a substantial portion of the HSL in unstimulated cells is soluble.

Khoo *et al.* (18) observed that stimulation of rat adipocytes produced a small drop in supernatant lipolytic activity (56% to 43%) that was accompanied by a small gain in lipid-associated lipase activity. By contrast, we observed a near-total loss of HSL activity in the supernatant upon stimulation, and Western blotting revealed a quantitative shift of the enzyme to the fat cake (Fig. 3). The greater magnitude of change in the present study probably reflects the use of PIA to ensure that control cells were indeed lipolytically unstimulated (11, 19). Interestingly, the finding that the lipase is transferred quantitatively suggests that the amount of the enzyme may be a rate-limiting factor in lipolysis. Thus, differences in lipolytic activities among adipocyte populations derived from various sources might be explained in part by differences in the mass of HSL as well as by differences in susceptibility to various receptor-mediated stimulators and inhibitors.

A clear consequence of lipolytic stimulation is that the enzyme binds tightly to the lipid particle, presumably anchored to a component at the periphery of the lipid storage droplet. In similar experiments on cultured 3T3-L1 adipocytes by Hirsch and Rosen (6), lipolytic stimulation caused the lipase to relocate from the soluble to the sedimented particulate fraction. In contrast to mature primary adipocytes, which contain a large unilocular lipid droplet, cultured 3T3-L1 cells contain considerable triacylglycerol in much smaller lipid storage droplets, many of which may sediment with membranes during centrifugation. Indeed, prevention of lipid synthesis by biotin deficiency eliminated the apparent translocation in the cultured cells. Although Hirsch and Rosen (6) did not examine the floating fat cake, their data on the cultured cells are consonant with ours on the primary adipocytes.

Our results may provide a molecular explanation for early findings of Vaughan *et al.* (20) that hormonal stimulation led to increased hydrolytic activity associated with the fat cake, an activity directed exclusively against endogenous lipid which persisted after extraction of the bulk lipid with petroleum ether (21). Oschry and Shapiro (22) found that

sonication of the fat cake from stimulated cells elicited hydrolytic activity of sufficient magnitude to explain the cellular response to adrenergic stimulation. The tight binding of the enzyme to the lipid droplet in stimulated cells presents an enigma, especially since the primary structure of HSL shows no regions of high hydrophobicity (4) and in preliminary experiments we have found no propensity of purified, phosphorylated HSL to bind to homogenized fat cakes from rat adipocytes. Wise and Jungas (23) proposed that the cellular lipolytic response involves "substrate activation" in concert with activation of the lipase—i.e., the lipid droplet surface may be more than a passive participant in the lipolytic process. We recently identified an adipocyte-specific protein, perilipin, that resides on the surface of the lipid droplet and, perhaps more importantly, is phosphorylated by A-kinase in parallel with activation of lipolysis (8, 9). Perilipin may qualify as an attractive candidate as a substrate-associated factor that participates in cellular lipolysis.

Although it has been reported that exogenous phosphorylation by A-kinase increases activity of the rat enzyme, usually ≤2-fold (1, 2, 6), the only positive effect we elicited upon phosphorylation was that the enzyme decayed more slowly (unpublished work). Overall, the data are compatible with the idea that HSL is constitutively active but inaccessible to cellular substrate unless cAMP is increased. Indeed, Stralfors *et al.* (2) found that the great lipolytic difference between stimulated and unstimulated intact cells largely disappeared after sonication of cells, primarily due to an increase in the activity of the sonicated unstimulated cells. Similar findings were reported by Salers *et al.* (24) on activities in homogenates of control and stimulated cells. Most recently, the elegant studies of Okuda *et al.* (25) demonstrated that disruption of lipid droplets led to maximal lipolytic activity irrespective of changes in cAMP, prompting the suggestion that the physicochemical character of the droplet plays an important role in the lipolytic process. Stralfors *et al.* (2) proposed that sonication increased accessibility to substrate by dispersing the lipid droplet and thus increasing the effective substrate concentration by increasing lipid surface area. Implicit in this explanation, which is reasonable, is the view that unphosphorylated HSL is highly, if not maximally active, raising the question on how phosphorylation alters lipolysis in the intact cell. Our results indicate that phosphorylation increases access to substrate by movement of the enzyme to the lipid storage droplet or by tighter binding of the enzyme to a component of the droplet surface.

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