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The Central Role of Perilipin A in Lipid Metabolism and Adipocyte Lipolysis

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Functional Studies on Native and Mutated Forms of Perilipins

A ROLE IN PROTEIN KINASE A-MEDIATED LIPOLYSIS OF TRIACYLGLYCEROLS IN CHINESE HAMSTER OVARY CELLS*

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Perilipin A coats the lipid storage droplets in adipocytes and is polyphosphorylated by protein kinase A (PKA); the fact that PKA activates lipolysis in adipocytes suggests a role for perilipins in this process. To assess whether perilipins participate directly in PKA-mediated lipolysis, we have expressed constructs coding for native and mutated forms of the two major splice variants of the perilipin gene, perilipins A and B, in Chinese hamster ovary fibroblasts. Perilipins localize to lipid droplet surfaces and displace the adipose differentiation-related protein that normally coats the droplets in these cells. Perilipin A inhibits triacylglycerol hydrolysis by 87% when PKA is quiescent, but activation of PKA and phosphorylation of perilipin A engenders a 7-fold lipolytic activation. Mutation of PKA sites within the N-terminal region of perilipin abrogates the PKA-mediated lipolytic response. In contrast, perilipin B exerts only minimal protection against lipolysis and is unresponsive to PKA activation. Since Chinese hamster ovary cells contain no PKA-activated lipase, we conclude that the expression of perilipin A alone is sufficient to confer PKA-mediated lipolysis in these cells. Moreover, the data indicate that the unique C-terminal portion of perilipin A is responsible for its protection against lipolysis and that phosphorylation at the N-terminal PKA sites attenuates this protective effect.

Acute mobilization of adipose triacylglyerol stores for energy is regulated primarily by the activation state of cAMP-dependent protein kinase (PKA)[1] (1). Historically, this stimulation has been attributed to phosphorylation and activation of hormone-sensitive lipase (HSL), but as noted previously (2, 3), the meager doubling of HSL activity upon phosphorylation *in vitro* falls far short of explaining the 30–100-fold activation of cellular lipolysis upon elevation of PKA activity in isolated primary adipocytes. Although some the of differences between the magnitude of the *in vitro* and *in vivo* responses may be attributed to the PKA-induced translocation of HSL from the cytosol to the lipid storage droplets within adipocytes (4), it is likely that additional factors, notably the perilipins, contribute to the cellular response.

The perilipins are a class of proteins found exclusively at the limiting surface of lipid storage droplets, *i.e.* at the lipid/aqueous interface, in adipocytes and in steroidogenic cells (5–7). These proteins are the most abundant PKA substrates in adipocytes (5), and both their subcellular location and polyphosphorylation by PKA suggest a role for the perilipins in PKA-mediated lipolysis. In adipocytes, alternative mRNA splicing gives rise to perilipins A and B, the former at much higher levels than the latter (8). Thus far, functional studies point to a role for the perilipins in protecting stored TAG from hydrolysis by cellular lipases. Ectopic expression of perilipin A in 3T3-L1 adipoblasts results in perilipin A-coated lipid droplets and increases the half-life of stored TAG deposits by a factor of 4.5 (9). Also, treatment of 3T3-L1 adipocytes with tumor necrosis factor-α both reduces expression of perilipin A and increases basal lipolysis. With the use of an adenovirus vector to maintain perilipin expression during tumor necrosis factor-α treatment, Souza *et al.* (10) found that expression of either perilipin A or B was sufficient to maintain a coating of perilipin on the droplets and to suppress tumor necrosis factor-α-activated lipolysis. Such findings further support the conclusion that perilipins can protect the intracellular TAG from hydrolysis by lipases. Finally, two reports describing the phenotype of the perilipin null (11, 12) mouse show that these animals have greatly reduced adipose stores and constitutively high levels of basal lipolysis in their isolated adipose cells, again consistent with the loss of the protective effect of perilipin. In one of these reports (12), it was shown that in the absence of perilipin, the adipose cells droplets were coated with adipose differentiation-related protein (ADRP), indicating that this related protein did not substitute for perilipin in providing a protective barrier against lipolysis in the absence of PKA activation.

None of the above studies address a role for phosphorylation of perilipin in the lipolytic response. To this end, using CHO cells, we have examined the functional consequences of introducing perilipin A, perilipin B, and mutant variants of these species. We report herein that the expression of perilipin A alone is sufficient to inhibit lipolysis by 87% and that upon activation of PKA, lipolysis is stimulated by 7-fold. These results re-create the strong regulatory role for perilipin seen in adipocytes and contrast with results from another system that detected only a modest, ~50% stimulation by PKA upon introduction of perilipin A into cells (13). We further show that such

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[1] The abbreviations used are: PKA, cAMP-dependent protein kinase; Peri, perilipin; Peri A, perilipin A; Peri B, perilipin B; ADRP, adipose differentiation-related protein; IBMX, isobutylmethylxanthine; TAG, triacylglycerol; HSL, hormone-sensitive lipase; E-MLV, ecotropic murine leukemia virus.

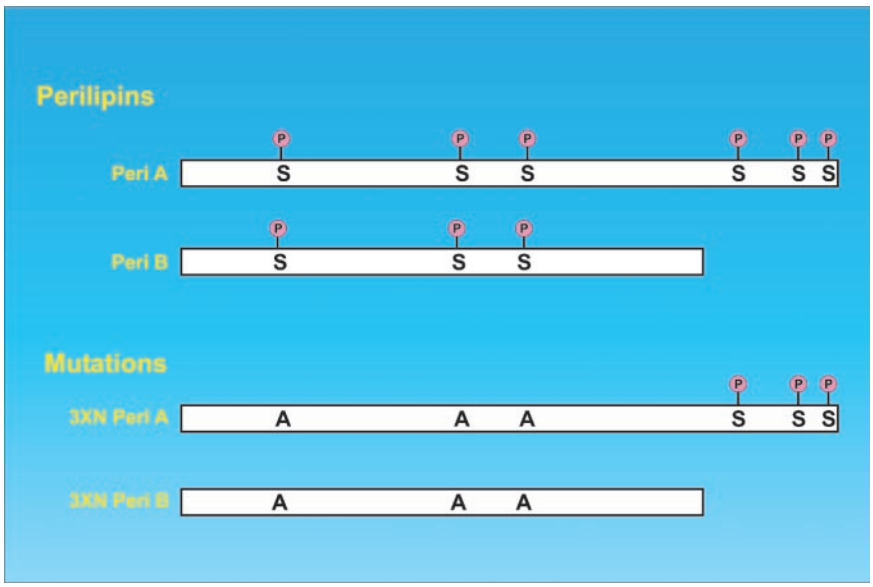

FIG. 1. **The various constructs produced for this study are depicted.** *Peri A*, full-length perilipin A; *3XN Peri A*, perilipin A with serines in three N-terminal PKA sites at amino acids 81, 222, and 276 mutated to alanines; *Peri B*, full-length Peri B; *3XN Peri B*, perilipin B with Ser-to-Ala mutations at residues within PKA sites at amino acids 81, 222, and 276.

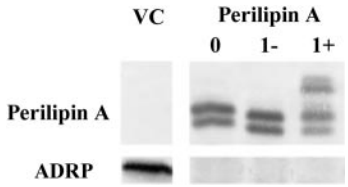

FIG. 2. **Shift in perilipin A migration under SDS-PAGE following stimulation with IBMX and forskolin.** Cells expressing perilipin A and vector control (*VC*) cells were either unstimulated *(lane 1−)* or stimulated (*lane 1+*) with 1 mM IBMX and 10 μM forskolin for 10 min. Homogenates were subjected to SDS-PAGE and immunoblotted with anti-perilipin and anti-ADRP antisera. The characteristic upward shift of PKA-phosphorylated perilipin (*PA*) in stimulated cells is evident.

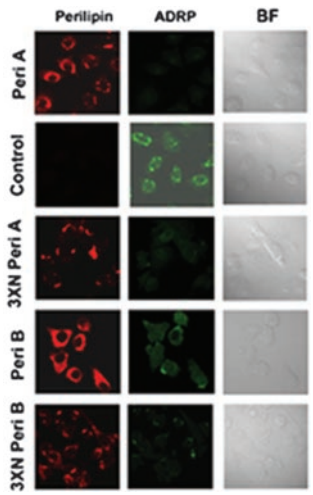

FIG. 3. **Immunofluorescence of different cell types with anti-perilipin and anti-ADRP.** Cells were infected with retroviral constructs bearing the indicated forms of perilipin. Abbreviations are described in the legend for Fig. 1 except for *BF*, which indicates bright field. *Control* indicates the empty vector with no perilipin insert. Cells were plated, grown, and loaded with lipid, fixed, and immunostained for perilipin and ADRP as described under "Experimental Procedures."

regulation is abrogated upon mutation of the PKA sites that lie within the N-terminal region of perilipin, as did Souza *et al.* (13). In contrast, we report that neither native nor mutated perilipin B provide protection.

EXPERIMENTAL PROCEDURES

Expression of Perilipins in CHO Cells—Perilipin functional studies were conducted with CHO K-1 cells (American Type Culture Collection, Manassas, VA) into which we introduced murine perilipins A and B and mutated forms of these proteins in which serine residues within consensus PKA sites were mutated to alanines. Mutations were made in sets of three, by grouping the three N-terminal PKA sites (amino acids 81, 222, and 276). The accession number for murine perilipin A cDNA is GenBank™ AY161165. The terminologies for the various constructs are shown in Fig 1. Each mutation was made by PCR using primers containing the mutation of interest and external primers to amplify a small cassette, which was then cloned in-frame in mouse perilipin A cDNA (14). The accuracy of each mutation was confirmed by sequencing. Both unmodified and mutant full-length cDNAs were cloned in the retroviral expression vector, pSR MSV-Tk Neo. Retrovirus was produced by co-transfecting retroviral expression vectors and the packaging vector, pSV ψ E-MLV (15), into HEK-293 T fibroblasts using calcium phosphate. Cells were transfected for 9 h in the presence of chloramphenicol, following which the medium was changed to fresh Dulbecco's modified Eagle's medium, and viral production was continued for 48 h. Prior to infection, CHO-K1 fibroblasts were treated overnight with tunicamycin (16, 17) to increase the infection rate. Cells were infected for 24 h using medium containing 4 μg/ml polybrene harvested from the 293 T cells. Immediately following infection, CHO cells were placed under selection in 600 μg/ml G418.

Lipid Loading and Hydrolysis—Cells infected with retrovirus-containing perilipin inserts or control cells, which stably incorporated the retrovirus vector lacking perilipin cDNA, were plated at a density of 0.5×10^6 cells/35-mm (diameter) well. After cells became adherent, the medium was changed to F-12 (Invitrogen) supplemented with 400 μM oleic acid complexed to 0.4% bovine serum albumin to promote triacylglycerol deposition. [^{3}H]Oleic acid, at 1×10^6 dpm/well, was included as a tracer. Following a 24-h loading period, the cells were washed with 4% bovine serum albumin in PBS to remove unincorporated oleic acid. These conditions were employed for all experiments in the present studies, and the amount of protein per well did not differ among cells bearing the different perilipin species; thus, data are expressed on a per well basis. For lipid analysis, the cells were extracted with chloroform:methanol (2:1) (18), and lipids were identified by thin layer chromatography on silica gel plates (Analtech, Newark, DE). Mobile phase 1 was acetone developed for 4 cm; mobile phase 2 was 80:20:1 petroleum ether:diethyl ether:acetic acid developed to the top of the plate (19). To measure hydrolysis of cellular lipids, cells were loaded with radiolabeled oleic acid as described above and placed in an efflux medium, which was F-12 medium including 1% defatted bovine serum albumin (ICN Biomedical, Aurora, OH) as a fatty acid acceptor. The efflux of radioactivity to the medium was measured over time by scintillation counting. TLC confirmed that all radioactivity released to the medium was free [^{3}H]oleic acid. Re-esterification of fatty acids was prevented by the inclusion of 2.5 μM Triacsin C (Biomol, Plymouth Meeting, PA), an inhibitor of acyl co-enzyme A synthetase, to the medium. In preliminary experiments, this level of Triacsin C was found to effectively block all fatty acid esterification in CHO cells. To

stimulate protein kinase A activity, cAMP levels in cells were elevated by the addition of 1 mM isobutylmethylxanthine (IBMX) and 10 μM forskolin. The efficacy of this stimulation method was confirmed by immunoblotting, which revealed that upon stimulation of cells that were expressing perilipin A, the protein migrated more slowly under SDS-PAGE, characteristic of PKA-phosphorylated perilipin A (Fig. 2).

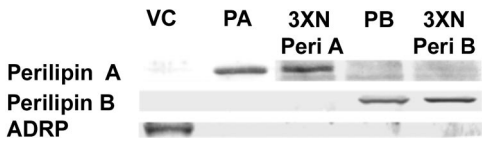

FIG. 4. **Immunoblot analysis of different types of cells.** Cells from Fig. 3 were homogenized, subjected to SDS-PAGE, and stained for perilipin and ADRP. Abbreviations are as described in legend for Fig 1. *VC*, vector control; *PA*, perilipin A; *PB*, perilipin B.

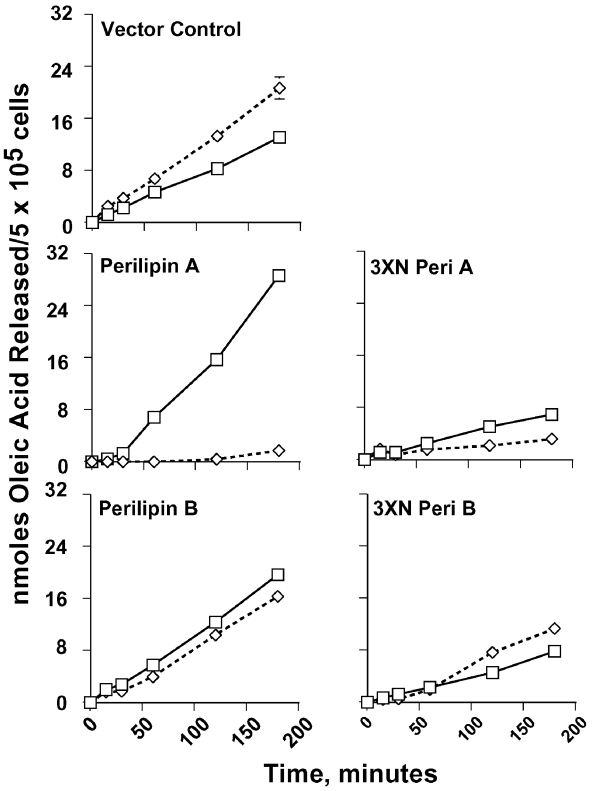

FIG. 5. **Time courses of [^{3}H]oleic acid release under unstimulated and stimulated conditions.** Cells bearing the indicated constructs were loaded with [^{3}H]oleic acid as described under "Experimental Procedures," and the efflux of [^{3}H]oleic acid to the medium was tracked over 3 h. Unstimulated cells received no further treatment, whereas stimulated cells were treated with 1 mM IBMX and 10 μM isoproterenol.

RESULTS

As we reported previously (20), lipid droplets in CHO cells are coated with ADRP (Figs. 2 and 3); this protein and the perilipins share significant sequence homology over their N-terminal domains (7). Upon expression of constructs that encode either native or mutated perilipin A, as well as native or mutated perilipin B, the droplets acquired a coating of perilipin and the ADRP diminished, as evidenced both by immunostaining (Fig. 3) and immunoblotting (Figs. 2 and 4). This change in the lipid droplet coat protein is a reprise of the perilipin-for-ADRP switch that occurs during differentiation of 3T3-L1 adipocytes (20). Moreover, the amounts of unmodified and mutated perilipins A and B were highly similar. The only cell lines carried forth to the metabolic studies below were those in which the perilipin (A or B) expression was sufficient to eliminate all ADRP expression, as judged both by immunofluorescence and by immunoblotting. Upon repeated passage of some perilipin A-expressing cell lines, the perilipin expression waned, and the metabolic phenotype reverted to that similar to the control cells with ADRP-coated droplets. In general, a reversal of the perilipin A phenotype occurred when the perilipin expression declined by ~50%.

To assess lipolytic activity, CHO cells were loaded with [^{3}H]oleic acids as described previously (9), after which the efflux of [^{3}H]oleic acid to the medium was monitored for 3 h. Triacsin C was included in all incubations during the efflux phase to prevent reutilization of fatty acids released from hydrolyzed TAG (21–23). In control cells (with ADRP-coated droplets), nearly 30% of the stored [^{3}H]oleic acid was released to the medium over 3 h, and PKA activation inhibited fatty acid efflux by about 30%. In contrast, in cells expressing perilipin A, in the absence of PKA activation, the release of [^{3}H]oleic acid was suppressed by 87% when compared with that in control cells with ADRP-coated lipid droplets. Upon activation of PKA with the combination of forskolin and IBMX, lipolysis was activated by 7-fold (range of 3–13-fold) over the unstimulated cells. Iin Fig. 5 (*Perilipin A panel*), note that the stimulatory effects of PKA activation on oleic acid release in Peri A cells occurred only after a lag of ~30 min. During this 30-min period, there were no differences in the rates of oleic acid release in unstimulated and stimulated cells; thereafter, lipolysis under the two conditions diverged and remained nearly linear over the next 2.5 h. There was another highly reproducible break in the curve at 120 min, indicating that the perilipin-mediated increase in lipolysis is a slowly evolving process. The lipolytic rates for all

TABLE I

Lipolysis in CHO cells transfected the indicated perilipin constructs

Cells were loaded with radiolabeled oleic acid as described under "Experimental Procedures" and subsequently, the rates of oleic acid release were measured from 30 min until 3 h in unstimulated cells and in cells stimulated with the combination of IBMX and forskolin. The values shown for lipolytic rates are the averages of individual rates calculated for 3–6 experiments (3–6 dishes per experiment). Linearity of rates was assessed by linear regression analysis, and r-squared vales were calculated using Cricket Graph III. Significance among different groups was determined by using a paired Student's *t* test (Microsoft Excel) (27).

Lipid droplets coated with	Unstimulated	r^2	Stimulated	r^2
	nmol oleic a./h		*nmol oleic a./h*	
Control/ADRP[a]	7.85	.998	4.85	.996
Peri A[b]	1.08	.847	8.04	.993
Peri A 3XN[c]	1.13	.976	1.95	.994
Peri B[d]	4.39	.998	5.92	.999
Peri B 3X[e]	2.52	.989	2.33	.990

[a] For differences between unstimulated and stimulated results, *P* value is 0.0003.
[b] For differences between unstimulated and stimulated results, *P* value is 0.01.
[c] For differences between unstimulated and stimulated results, *P* value is 0.03.
[d] For differences between unstimulated and stimulated results, *P* value is 0.07 (NS); NS = not significant.
[e] For differences between unstimulated and stimulated results, *P* value is 0.35 (NS); NS = not significant.

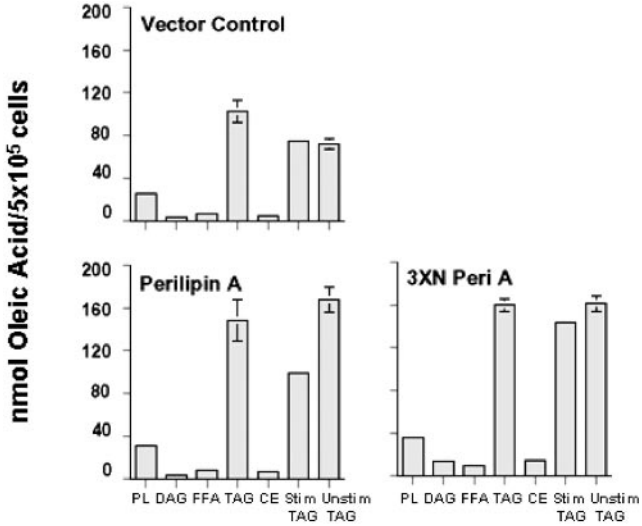

FIG. 6. **Distribution of [^{3}H]oleic acid among lipid pools in loaded CHO cells.** Bars labeled *PL* (phospholipid), *DG* (diacylglycerol), *FFA* (free fatty acid), *TAG*, and *CE* (cholesteryl ester) were analyzed immediately following the loading phase. *Unstim TAG* and *Stim TAG* represent analysis of triacylglycerol following 3 h of incubation under unstimulated and stimulated conditions, respectively.

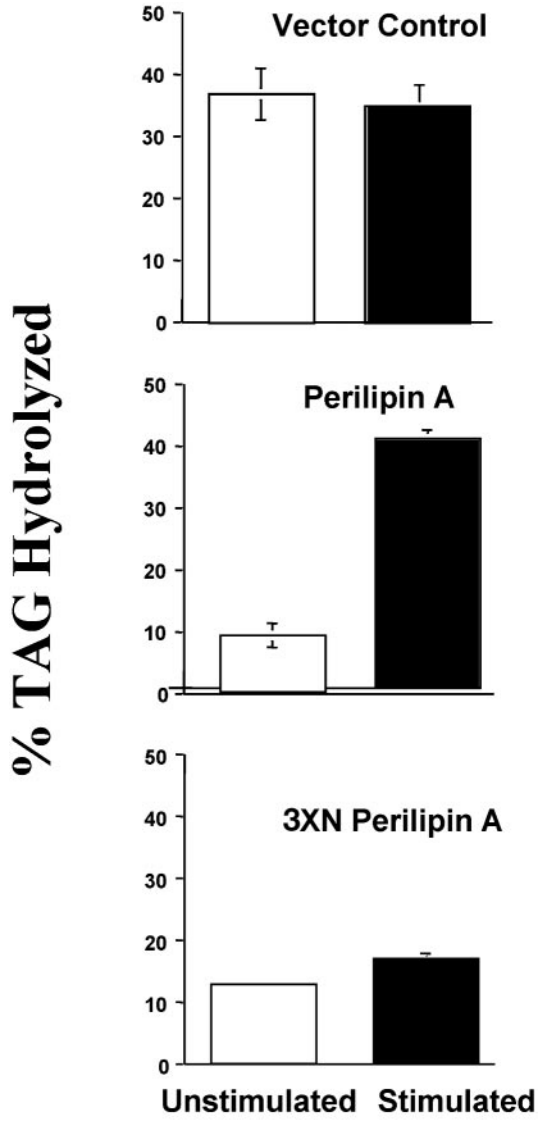

FIG. 7. **Percentages of TAG hydrolyzed over 3 h from control, Peri A, and 3 XN Peri A cells.** Cells were loaded with oleic acid as described under "Experimental Procedures." Radiolabeled TAG was measured immediately after loading and after a subsequent 3-h incubation with and without stimulation with IBMX and forskolin. *Bars* represent percentages of TAG hydrolyzed over 3 h for each condition; *error bars* represent mean values ± S.E. (n = 6).

constructs tested over the 2.5-h period are depicted in Table I. The 30-min lag was evident in all cells infected with perilipin species that were responsive to PKA activation. The paradoxical inhibition of oleic acid release upon PKA activation of control cells occurred without a detectable lag, as shown in Fig. 4 (*Vector Control Panel*). Perilipin A from mouse contains six consensus PKA phosphorylation sites (Fig. 1). We also tested a mutant variant of perilipin A in which the three most N-terminal serines within PKA sites were mutated to alanines (Peri 3XN). Introduction of this form of perilipin A also prevented the release of radiolabeled fatty acid stores in the absence of PKA activation, but unlike native perilipin A, the mutant form was relatively unresponsive to PKA activation. In contrast to the 7-fold increase with the perilipin A cells, the activation of the mutated perilipin was merely 1.7-fold (Table I). In contrast, perilipin B, the shorter splice variant of the perilipin gene failed to shield the TAG from hydrolysis and the native perilipin B, inhibiting activity by only 13%, when compared with the 90% inhibition by perilipin A. Also, perilipin B cells were unresponsive to PKA activation. Oddly, the mutated form of perilipin B (Peri B 3X) was more protective than native perilipin B, inhibiting lipolysis by 63% but unresponsive to PKA activation (Table I).

Under the loading conditions used in these studies, the great majority of the fatty acids taken up by the cells (>75%) were stored as TAG, and the distribution of tritiated oleic acid among the phospholipid, diacylglycerol, cholesteryl ester, and fatty acid pools was independent of the nature of coating on the lipid droplets (Fig. 6). However, the magnitude of uptake and storage of [^{3}H]oleic acid in the TAG pool was dependent on which protein coated the droplets. Cells expressing perilipin A accumulated the greatest amount, 148.9 ± 19.4 nmol oleic acid, greater than the perilipin B-expressing cells, 125.6 ± 3 nmol oleic acid, which, in turn, was greater than control cells with ADRP-coated droplets, 103.0 ± 10.3 nmol oleic acid (mean ± S.E., n = 6). These accumulations represent the steady state balance between lipogenesis and lipolysis during the lipid loading phase; accordingly, these levels reflect the differences in unstimulated lipolysis since the cells were loaded under the non-stimulated condition, *i.e.* no IBMX or forskolin. After the 3-h efflux incubation, the non-TAG pools lost no measurable [^{3}H]oleic acid (Fig. 6), whereas the TAG pools exhibited substantial losses, which again was dependent on the lipid droplet coat protein and the PKA activation state (Fig. 7). Moreover, TLC analysis revealed that all of the radiolabel that appeared in the medium was in the form of [^{3}H]oleic acid, which could be accounted for entirely by the loss of radiolabeled fatty acid from the intracellular TAG pool.

The percentages of TAG hydrolyzed over 3 h in control, Peri A, and Peri A 3XN cells are summarized in Fig. 7. It is clear that the TAG pool, presumably within the Peri A-coated pool, is the source of the accelerated oleic acid release under PKA-stimulation.

DISCUSSION

In the present study, we have demonstrated that expression of perilipin in CHO fibroblasts results in perilipin-coated lipid droplets from which the ADRP has disappeared. Perilipin A, but not perilipin B, suppresses lipolysis of droplet TAG, and PKA phosphorylation of perilipin A engenders a robust lipolytic reaction despite the absence of HSL or any other PKA-medi-

ated lipase. Unlike PKA-mediated lipolysis in adipocytes, the perilipin A-mediated lipolysis occurs only after a lag of ~30 min. Lipolysis is not stimulated when the serine residues within the three PKA sites at the N-terminal region of perilipin A are mutated to alanines.

The finding that the proteinaceous coating of the lipid droplets may confer PKA-mediated regulation of TAG hydrolysis prompts a reconsideration of the conventional view of PKA-mediated lipolysis in adipocytes. Heretofore, such activation was attributed primarily to the phosphorylation of HSL, following which the lipase was activated, but only minimally, and translocated from the cytosol to the lipid droplet surface. Clearly, given the above findings, one must account for the contribution of PKA phosphorylation of perilipin to the regulation of lipolysis. Interestingly, the *HSL* null mouse retains a modicum of isoproterenol-stimulated lipolytic activity, which led to the suggestion that adipocytes may contain a second hormone-sensitive lipase (24). Given the present results, plus the likelihood that the *HSL* null mouse expresses perilipins in its adipocytes, the presence of these lipid droplet-coating proteins may explain the residual PKA-activated lipolysis in the *HSL* null mouse (25).

Despite the earlier report suggesting that HSL may be expressed in CHO cells (26), we detected no HSL by immunoblotting the these cells nor could we detect any PKA-stimulated lipolytic activity in CHO cell homogenates (data not shown). Thus, the neutral lipid lipase responsible for the TAG hydrolysis in the CHO cells appears unresponsive to PKA, and it is concluded that PKA regulation of lipolysis in CHO cells expressing perilipin A results solely from the PKA phosphorylation of the perilipin. Clearly, the ability of the endogenous CHO cell lipase(s) to hydrolyze stored TAG under the PKA-stimulated condition is dependent on the phosphorylation of perilipin A. This conclusion is supported by the finding that mutation of selected PKA sites eliminated the regulated response. In a separate study, we show that perilipin A acts cooperatively with HSL in lipolysis when both proteins are introduced into CHO cells.[2] Thus, the ability of perilipin to regulate lipolysis of the TAG within lipid droplets appears to be manifested with any lipase that has access to the lipid droplet surface. The enhancement of lipolytic activity by perilipin A and the endogenous lipase of CHO cells is clearly a time-dependent process that requires at least 30 min after stimulation to become manifest. In contrast, when both HSL and perilipin are introduced into CHO cells, the lipolytic activation is nearly immediate with no detectable lag.[2] We assume that the phosphorylation of HSL fosters this immediate reaction in contrast to the endogenous lipase, which is apparently not a PKA substrate. Oddly, perilipin B neither protected against lipolysis nor conferred significant PKA regulation of lipolysis, which was not anticipated given the results of Souza *et al.* (10). However, in the present study, we expressed perilipin B without perilipin A, whereas in the studies of Souza *et al.* (10), perilipin B was introduced into cells that contained abundant perilipin A.

The fatty acids that appeared in the CHO cell medium in the present study derived from the triolein stored in the intracellular neutral lipid droplets, which were coated with either ADRP or the perilipins introduced into the cells. No other lipid pool contributed to the fatty acid that was released. Moreover, stimulation of cells expressing perilipin A led to a loss solely from the triolein from within the perilipin-coated droplets. Thus, one may assume that the PKA-mediated increase in fatty acid release stems from changes in the lipid droplet surface upon phosphorylation of perilipin A, a conclusion supported by the finding that the 3XN mutant species failed to respond to PKA activation.

The present data permit speculation on the structural determinants for perilipin function at the lipid droplet surface. If perilipins A and B assume similar extended conformations at the droplet surface, the greater protection against hydrolysis of the longer splice variant, perilipin A, may be attributed to its greater length and thus greater coverage of droplet surface, *i.e.* it is the extended C-terminal tail (~12 kDa) of perilipin A that is important in protecting against lipolysis in the unstimulated state. It follows that phosphorylation of PKA sites in the N-terminal region induces a conformational change in the C-terminal region that exposes portions of the lipid droplet surface to lipase action under conditions of PKA activation.

In addition to the present studies on CHO cells, it is reasonable to assume that phosphorylation of perilipin contributes to the lipolytic activation in adipocytes. This conclusion is reinforced by the finding that lipolytic stimulation by β-adrenergic agonists of adipocytes from perilipin null mice is strongly blunted despite the presence of normal amounts of HSL (12).

After completion of the present studies, a similar study appeared that largely agrees with a number of the findings reported herein (13), but that study did not include studies with perilipin B, and mutational analysis was limited to the construct we term Peri A 3XN (13). Although the present studies and those of Souza *et al.* (13) agree qualitatively, there are large quantitative differences between the two studies. For example, we find that introduction of perilipin A suppresses lipolysis by ~90% when compared with control cells, whereas Souza *et al.* (13) observed a suppression of about 50%. Also, we find that activation of Peri A cells leads to 162% greater lipolysis than in control cells, as compared with a 50% increment in the other study. Further, PKA activation of perilipin A cells produced a 700% increase in lipolysis, as compared with a 160% increase in the published study. Our findings suggest that PKA phosphorylation of perilipin A does far more than merely abrogate the protective effect of the perilipin, as suggested by Souza *et al.* (13), but additionally, exposes considerably more TAG to lipase activity than is exposed in the control cells containing droplets coated with ADRP. Reasons for these differences may include the use by Souza *et al.* (13) of NIH 3T3 fibroblasts engineered to be more lipogenic by using cells that also expressed an ectopic fatty acid transporter and an ectopic acyl synthase, whereas in the present studies, we used an acyl synthase inhibitor to block re-esterification of fatty acids. Also, we used CHO cells that were stably transfected with the various perilipin constructs, whereas Souza *et al.* (13) infected the cells with perilipin adenovirus. In separate studies, we, too, used adenovirus to introduce perilipin into cells and find modest effects with CHO cells similar to those reported by Souza *et al.* (13) for NIH 3T3 cells.[2]

In parallel studies, we demonstrate both that HSL translocation to lipid droplets requires that such droplets be coated with PKA-phosphorylatable perilipin A[2] and that HSL be phosphorylated at one of its C-terminal PKA sites, Ser-659 or 660.[3] Thus, the physiological activation lipolysis in adipocytes is a concerted reaction between phospho-HSL and phospho-perilipin.

REFERENCES

1. Honnor, R. C., Dhillon, G. S., and Londos, C. (1985) *J. Biol. Chem.* **260,** 15130–15138
2. Londos, C., Brasaemle, D. L., Schultz, C. J., Adler-Wailes, D. C., Levin, D. M., Kimmel, A. R., and Rondinone, C. M. (1999) *Ann. N. Y. Acad. Sci.* **892,**

[2] C. Sztalryd, G. D. H. Xu, A. R. Kimmel, and C. Londos, submitted for publication.

[3] C.-L. Su, C. Sztalryd, J. A. Contreras, C. Holm, A. R. Kimmel, and C. Londos, submitted for publication.

155–168
3. Holm, C., Osterlund, T., Laurell, H., and Contreras, J. A. (2000) *Annu. Rev. Nutr.* **20,** 365–393
4. Brasaemle, D. L., Levin, D. M., Adler-Wailes, D. C., and Londos, C. (1999) *Biochim. Biophys. Acta* **1483,** 251–262
5. Greenberg, A. S., Egan, J. J., Wek, S. A., Garty, N. B., Blanchette-Mackie, E. J., and Londos, C. (1991) *J. Biol. Chem.* **266,** 11341–11346
6. Servetnick, D. A., Brasaemle, D. L., Gruia-Gray, J., Kimmel, A. R., Wolff, J., and Londos, C. (1995) *J. Biol. Chem.* **270,** 16970–16973
7. Londos, C., Brasaemle, D. L., Schultz, C. J., Segrest, J. P., and Kimmel, A. R. (1999) *Semin. Cell Dev. Biol.* **10,** 51–58
8. Greenberg, A. S., Egan, J. J., Wek, S. A., Moos, M. C., Jr., Londos, C., and Kimmel, A. R. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 12035–12039
9. Brasaemle, D. L., Rubin, B., Harten, I. A., Gruia-Gray, J., Kimmel, A. R., and Londos, C. (2000) *J. Biol. Chem.* **275,** 38486–38493
10. Souza, S. C., de Vargas, L. M., Yamamoto, M. T., Lien, P., Franciosa, M. D., Moss, L. G., and Greenberg, A. S. (1998) *J. Biol. Chem.* **273,** 24665–24669
11. Martinez-Botas, J., Anderson, J. B., Tessier, D., Lapillone, A., Chang, B. H. J., Quast, M. J., Forenstein, D., Chen, K. H., and Chan, L. (2000) *Nat. Genet.* **26,** 474–479
12. Tansey, J. T., Sztalryd, C., Gruia-Gray, J., Roush, D. L., Zee, J. V., Gavrilova, O., Reitman, M. L., Deng, C. X., Li, C., Kimmel, A. R., and Londos, C. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 6494–6499
13. Souza, S. C., Muliro, K. V., Liscum, L., Lien, P., Yamamoto, M. T., Schaffer, J. E., Dallal, G. E., Wang, X., Kraemer, F. B., Obin, M., and Greenberg, A. S. (2002) *J. Biol. Chem.* **277,** 8267–8272
14. Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., and Erlich, H. A. (1988) *Science* **239,** 487–491
15. Muller, A. J., Young, J. C., Pendergast, A. M., Pondel, M., Landau, N. R., Littman, D. R., and Witte, O. N. (1991) *Mol. Cell. Biol.* **11,** 1785–1792
16. Wilson, C. A., and Eiden, M. V. (1991) *J. Virol.* **65,** 5975–5982
17. Miller, D. G., and Miller, A. D. (1992) *J. Virol.* **66,** 78–84
18. Bligh, E. G., and Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37,** 911–917
19. Levacher, C., Sztalryd, C., and Picon, L. (1985) *Reprod. Nutr. Dev.* **25,** 169–173
20. Brasaemle, D. L., Barber, T., Wolins, N. E., Serrero, G., Blanchette-Mackie, E. J., and Londos, C. (1997) *J. Lipid Res.* **38,** 2249–2263
21. Igal, R. A., Wang, P., and Coleman, R. A. (1997) *Biochem. J.* **324,** 529–534
22. Tomoda, H., Igarashi, K., Cyong, J. C., and Omura, S. (1991) *J. Biol. Chem.* **266,** 4214–4219
23. Tomoda, H., Igarashi, K., and Omura, S. (1987) *Biochim. Biophys. Acta* **921,** 595–598
24. Slatiel, A. R. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 535–537
25. Osuga, J., Ishibashi, S., Oka, T., Yagyu, H., Tozawa, R., Fujimoto, A., Shionoiri, F., Yahagi, N., Kraemer, F. B., Tsutsumi, O., and Yamada, N. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 787–792
26. Osuga, J., Ishibashi, S., Shimano, H., Inaba, T., Kawamura, M., Yazaki, Y., and Yamada, N. (1997) *Biochem. Biophys. Res. Commun.* **233,** 655–657
27. Student, R. (1908) *Biometrika* **6,** 1–25