Title page

Title:

Shrinking and Development of Lipid Droplets in Adipocytes during Catecholamine-induced Lipolysis

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Abstract:

Time-lapse observation of adipocytes during catecholamine-induced lipolysis clearly shows that shrinking of existing lipid droplets (LDs) occurs in some adipocytes and that small LDs are newly developed in almost all cells. Immunofluorescence imaging reveals that activation and localization of hormone-sensitive lipase (HSL) on the surface of LDs, which are required for conferring maximal lipolysis, are necessary for the shrinking of the LDs. However, not all adipocytes in which phosphorylated HSL is localized on LDs exhibit shrinking of LDs. The

simultaneous shrinking and development of LDs yield apparent fragmentation and dispersion of LDs in adipocytes stimulated with catecholamine.

Keywords:

Adipocytes; Lipid droplets; Norepinephrine; Adipose triglyceride lipase (ATGL); Hormone-sensitive lipase (HSL); Time-lapse observation

List of abbreviations:

ATGL, adipose triglyceride lipase; BSA, bovine serum albumin; DIC, differential interference contrast; FFA, free fatty acid; HSL, hormone-sensitive lipase; LD, lipid droplet; PBS, phosphate-buffered saline; PKA, protein kinase A; SVC, stromal-vascular cell; TG, triglyceride

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1. Introduction

The most characteristic organelle of adipocytes is the lipid droplet (LD), in which triglyceride (TG) is stored. Numerous studies have revealed that LDs act as highly dynamic organelles that play a crucial role in regulating energy homeostasis rather than as an inert reservoir for storing TG (for reviews, see [1-3]); in other words, mobilization of TG stored in LDs is also a major function of adipocytes. Stimulation of adipocytes with catecholamine promotes TG hydrolysis (lipolysis) and greatly increases the extracellular release of free fatty acid (FFA) and glycerol from adipocytes [4]. Catecholamine stimulation induces translocation of hormone-sensitive lipase (HSL), one of the major lipases in adipocytes, from the cytosol to the LD surface to allow HSL to interact with LDs [4,5]. The translocation and lipase activities of HSL are regulated by protein kinase A (PKA)-dependent phosphorylation of multiple sites [6] (for review, see [7]). Perilipin, a LD-associated protein, acts as a barrier that prevents cytosolic HSL from interacting with LDs in a basal (unstimulated) state [8]. Perilipin A contains multiple PKA phosphorylation sites. Catecholamine-induced phosphorylation of perilipin A promotes LD dispersion and lipolysis, the latter being caused by an interaction between perilipin A and phosphorylated HSL on the LD surface [9-11]. Adipose triglyceride lipase (ATGL) was identified as another TG lipase that acts in conjunction with HSL to hydrolyze TG [12]. ATGL activation by CGI-58 is required for the efficient TG lipase activity of ATGL [13-15]. CGI-58 can also interact with unphosphorylated, but not phosphorylated, perilipin [15].

To date, the complicated molecular processes involved in lipolysis have been extensively studied, as mentioned above (for review, see [16]); however, there are only few studies on the morphological changes of LDs in adipocytes during catecholamine-induced lipolysis. Brasaemle and colleagues performed immunofluorescence imaging of perilipin and found that fragmentation and dispersion of LDs are caused by prolonged lipolytic stimulation [9,17,18]. Recently, it was reported that micro-LDs form in cytoplasmic regions that are distant from the existing LDs within 10 min after catecholamine stimulation [15]. However, whether the LDs shrink or are fragmented into several smaller ones in catecholamine-stimulated adipocytes remains controversial. We previously revealed temporal and spatial variations in LDs in individual adipocytes during adipocyte division and differentiation by time-lapse microscopy [19]. Here we show morphological changes of LDs in adipocytes during catecholamine-induced lipolysis and clarify the relationship between these changes and subcellular localization of lipases.

2. Materials and Methods

2.1. Cells and cell culture

The study used adipocytes differentiated from stromal-vascular cells (SVCs) in vitro. SVCs

were harvested from the mesenteric adipose tissue of male Sprague-Dawley rats at 3-5 weeks of age as described previously [20]. The SVCs were seeded at approximately 3×10^4 cells/cm^2 in coverslip-bottom dishes and cultured in a culture medium consisting of DMEM/F12 supplemented with 10% newborn calf serum, 17 μM pantothenic acid, 33 μM biotin, 100 μM ascorbic acid, 1 μM octanoic acid, 50 nM triiodothyronine, 2.5 mM niacinamide, 0.85 ng/ml insulin, 200 ng/ml insulin-like growth factor-1, 100 units/ml penicillin, and 100 μg/ml streptomycin. As reported previously [21], when the SVCs were cultured for more than 6 days with medium changes every other day, most of the cells differentiated into adipocytes containing numerous LDs. It should be noted that dexamethasone and 3-isobutyl-1-methylxanthine are not required for inducing adipocyte differentiation, and that insulin concentration in the culture medium, which corresponds to physiological levels, is significantly lower than that used in conventional methods.

2.2. Time-lapse observation

Time-lapse observation of catecholamine-induced morphological changes of LDs in living adipocytes was performed after 6 to 9 days in culture. For time-lapse observation, coverslip-bottom dishes in which differentiated adipocytes were cultured were completely filled with fresh culture medium with or without either 1×10^{-7} M norepinephrine (Sigma, St. Louis, MO, USA) or 1×10^{-7} M isoproterenol (Sigma) and sealed with a sterile coverslip and silicone

grease (Dow Corning Toray Co., Ltd., Tokyo, Japan) [19,22]. A sealed dish was placed on the sample stage of a differential interference contrast (DIC) microscope (IX81; Olympus, Tokyo, Japan) equipped with a temperature controller and a digital CCD camera (DP70; Olympus), after which time-lapse images were captured at 1-min intervals for 8 h or at 30-sec intervals for 2 h. The series of captured images was converted into a movie using MediaStudio Pro software (Ulead Systems, Inc., Yokohama, Japan).

2.3. Immunofluorescence imaging

Immunofluorescence imaging of HSL and ATGL was performed as follows. Cells were fixed with 4% formaldehyde in phosphate-buffered saline (PBS) for 10 min. The fixed cells were permeabilized with 0.01% digitonin (Sigma) in PBS for 10 min and incubated with 0.5% bovine serum albumin (BSA) in PBS for 30 min. Digitonin permeabilization less affects localization of LD-associated proteins in adipocytes than does Triton X-100 permeabilization [23]. The permeabilized cells were incubated for 1 h with either anti-phosphorylated HSL (Ser563) antibody (diluted 1:500; Cell Signaling Technology, Beverly, MA, USA) or anti-ATGL antibody (diluted 1:100; Cell Signaling Technology) in 0.5% BSA/PBS and then incubated for 1 h with 0.4% Alexa Fluor 488-labeled anti-rabbit IgG (Invitrogen/Molecular Probes, Carlsbad, CA, USA) in 0.5% BSA/PBS. Immunofluorescence staining of perilipin was also performed as described previously [19]. Fluorescence images and corresponding DIC images were taken

using a DIC microscope with epifluorescence optics (IX81; Olympus).

3. Results

3.1. Catecholamine-induced morphological changes of LDs

We performed time-lapse observation to investigate morphological changes of LDs during catecholamine-induced lipolysis. The observation was initiated immediately after differentiated adipocytes were stimulated with 1×10^{-7} M norepinephrine and continued for 8 h. Actual time-lapse images acquired at 1-min intervals were converted into a movie (see Movie 1 in supplementary data); the images extracted at 2-h intervals are shown in Fig. 1. Each differentiated adipocyte contained various numbers and sizes of LDs in its cytoplasm at 0 h. The existing LDs shrank considerably from immediately to 8 h after norepinephrine stimulation, and a part of them subsequently disappeared (*arrows* in Fig. 1). However, such shrinking of the LDs did not occur in all adipocytes. Adipocytes in which the LDs were preserved or enlarged were also observed (*arrowheads* in Fig. 1). Interestingly, although catecholamine stimulation would promote the release of FFA and glycerol from adipocytes, numerous small LDs (~1 μm) were newly developed in almost all the cells, including undifferentiated ones, in the latter half of the observation period.

We next performed time-lapse observation of adipocytes stimulated with 1×10^{-7} M isoproterenol, another catecholamine (Movie 2 in supplementary data). Morphological changes of LDs induced by isoproterenol stimulation were similar to those induced by norepinephrine stimulation in the following respects: existing LDs shrank in some adipocytes but were preserved in other adipocytes, and numerous small LDs developed in almost all the cells. In contrast, neither such shrinking nor development of LDs was observed in any cells when time-lapse observation of adipocytes without catecholamine stimulation was performed (data not shown).

3.2. Catecholamine-induced activation and translocation of lipases

To assess activation and translocation of HSL to the surface of LDs in response to catecholamine stimulation, we performed immunofluorescence staining of HSL phosphorylated at serine 563 in adipocytes at various time points after administration of 1×10^{-7} M norepinephrine (Fig. 2). Without norepinephrine stimulation, phosphorylated HSL was only slightly detected on the LD surface in some adipocytes (Fig. 2A). When adipocytes were stimulated with norepinephrine for 15 min or 2 h, phosphorylated HSL was strongly and exclusively localized on the LD surface in more than 60% of adipocytes (Figs. 2C and 2E). The phosphorylated HSL was clustered in a discontinuous and non-uniform pattern on the LD surface, and the intensity of the phosphorylated HSL staining varied among LDs contained in

individual adipocytes. In contrast, phosphorylated HSL was hardly detected when stimulated with norepinephrine for 8 h (Fig. 2G), being similar to that without stimulation.

We next assessed the expression and distribution of ATGL, another lipase present in adipocytes, by immunofluorescence staining (Fig. 3). Without norepinephrine stimulation, ATGL was expressed in all adipocytes and distributed in a punctate pattern throughout the cytoplasm rather than localized on the LD surface (Fig. 3A). Stimulation with 1×10^{-7} M norepinephrine for 2 h did not alter the expression or distribution of ATGL; that is, it did not induce significant translocation of ATGL from the cytoplasm to the LD surface in any adipocyte (Fig. 3C). Moreover, perilipin was localized exclusively on the surface of all LDs in any adipocyte, regardless of norepinephrine stimulation (data not shown).

We further revealed that stimulation with 1×10^{-7} M isoproterenol for 2 h induced extensive localization of phosphorylated HSL on the LD surface and did not alter the expression or distribution of ATGL (data not shown). These results are consistent with those of norepinephrine stimulation.

3.3. Relationship between shrinking of LDs and localization of phosphorylated HSL on LDs

To clarify the relationship between catecholamine-induced morphological changes of LDs and localization of phosphorylated HSL, we performed time-lapse observation at higher magnification of adipocytes stimulated with 1×10^{-7} M norepinephrine for 2 h and subsequent

immunofluorescence imaging of phosphorylated HSL in identical adipocytes. Time-lapse and immunofluorescence images of four adipocytes representative of 63 adipocytes in three experiments are shown in the left four columns (see also Movies 3 and 4 in supplementary data) and the right column of Fig. 4, respectively. Time-lapse observations showed that considerable shrinking of existing LDs occurred in 12 (19%) adipocytes (*arrowheads* in Figs. 4A and 4B), and that LD preservation or enlargement occurred in the other 51 (81%) adipocytes (Figs. 4C and 4D). Comparison of the behavior of LDs with localization of phosphorylated HSL in identical adipocytes revealed that phosphorylated HSL was strongly (11 adipocytes; for example, Figs. 4A and 4B) or moderately (one adipocytes) localized on the LD surface in all of the 12 adipocytes that exhibited considerable LD shrinking. In these adipocytes, the LDs that shrank were consistent with those on which phosphorylated HSL was localized. On the other hand, 18 adipocytes did not exhibit shrinking LDs, although phosphorylated HSL was strongly (Fig. 4C), moderately, or slightly localized on the LD surface in these adipocytes. Time-lapse observations also showed that numerous small LDs were newly developed and subsequently grew in the periphery of 56 (89%) adipocytes (*arrows* in Figs. 4A, 4C and 4D). Development of small LDs did not occur in the other seven adipocytes in which phosphorylated HSL was localized strongly or moderately on the existing LD surface (Fig. 4B). This finding implies that catecholamine-induced development of small LDs is at least partially suppressed in a competitive manner by HSL activation.

4. Discussion

Our time-lapse observations provided a detailed account of the morphological changes of LDs, such as shrinking and development, which occur in individual adipocytes during catecholamine-induced lipolysis (Figs. 1 and 4). The total or average lipolytic activity of all adipocytes in a culture dish is often determined by quantitatively measuring either the amount of FFA and glycerol released into the culture medium or the remaining amount of TG in adipocytes [4,8-11,13,14]. On the other hand, the time-lapse observation clearly shows the behavior of LDs in each adipocyte and thus allows us to evaluate differences between adipocytes in response to catecholamine.

A number of studies reported that ATGL plays critical roles in the hydrolysis of TG to diglyceride and in the determination of LD size in basal and stimulated lipolysis [12,24,25]. Thus, ATGL would be activated by catecholamine stimulation and contribute to lipolysis in the adipocytes used in this study. On the other hand, immunofluorescence imaging of HSL (Fig. 2) and ATGL (Fig. 3) showed that activation and translocation of HSL to the LD surface occurred during catecholamine-induced lipolysis, whereas translocation of ATGL did not occur. The finding suggests that in addition to ATGL activation, activation and localization of HSL on the surface of LDs, which are required for conferring maximal lipolysis, are necessary for the shrinking of LDs. Although no significant differences in the number of adipocytes showing

localization of phosphorylated HSL were seen between norepinephrine stimulation for 15 min and 2 h (Figs. 2C and 2E), the fluorescence intensity in the adipocytes stimulated for 2 h tended to be lower than those stimulated for 15 min. Considering that phosphorylated HSL was hardly detected in adipocytes stimulated with norepinephrine for 8 h as well as without it (Figs. 2A and 2G), these findings suggest that catecholamine-induced activation and translocation of HSL to the LD surface occur acutely and reach a maximal level within 2 h, and after which the levels gradually reduce and return to the control level within 8 h. The acute activation and translocation of HSL observed in this study is consistent with those in another study [4].

Time-lapse observation and immunofluorescence imaging showed that there are no significant differences in the behavior of LDs and endogenous lipases between stimulation with norepinephrine and stimulation with isoproterenol; the former is known as an α- and β-adrenergic agonist, while the latter is a pure β-adrenergic agonist. However, differences in the biochemical effects exerted by the two types of catecholamines on the adipocytes used in this study have not been assessed. Biochemical experiments, such as those measuring the elevation of cAMP levels and PKA activation, will thus be required for further discussion.

Examination of the relationship between LD behavior and localization of phosphorylated HSL in identical adipocytes revealed that localization of phosphorylated HSL on the LD surface is necessary for LD shrinking (Figs. 4A and 4B). However, not all the adipocytes in which phosphorylated HSL was localized on the LD surface exhibited LD shrinking (Fig. 4C). The timing of LD shrinking seems to be relatively later than that of the completion of HSL

activation and translocation (comparing Figs. 1 and 2). Taken together, these findings suggest that persistent rather than transient localization of phosphorylated HSL is required for LD shrinking. PKA-dependent phosphorylation of other sites of HSL and perilipin A would also be involved in regulating lipolysis as previously reported [6,10,11]. Further studies addressing localization of these active forms of HSL and perilipin A will be required to understand the molecular mechanisms underlying catecholamine-induced LD shrinking.

We finally discuss catecholamine-induced development and fragmentation of LDs. Numerous small LDs were newly developed and subsequently grew in almost all catecholamine-stimulated adipocytes (Figs. 1 and 4), as reported earlier [15]. In our time-lapse observations, however, LDs never divided into several smaller ones (Figs. 1 and 4). Alternatively, catecholamine stimulation simultaneously induces LD shrinking and development, and thus yields apparent LD fragmentation and dispersion in adipocytes (Fig. 4A). Considering that small LDs were also developed in undifferentiated fibroblast-like cells (Fig. 1), the development of small LDs is probably caused by an increase in the release of FFA from the adipocytes, that is, an elevation in FFA levels in culture medium. This is supported by previous findings that treatment with fatty acids promotes rapid accumulation of LDs in 3T3-L1 adipocytes [26], NIH 3T3 cells [27], and other cells [23,28-30]; however, our model is inconsistent with the previous finding that phosphorylation of serine 492 of perilipin A induces LD dispersion even when basal and stimulated lipolysis are completely inhibited [9]. It has been previously reported that LD formation induced by unsaturated fatty acids such as oleic acid and arachidonic acid is inhibited

by an inhibitor of microtubule assembly [27] and by inhibitors of protein kinase C, phosphoinositide 3-kinase, and p38 mitogen-activated protein kinase [31,32]; therefore, time-lapse observation of catecholamine-stimulated adipocytes pre-treated with these inhibitors would provide further clarification.

In conclusion, our time-lapse observations revealed the morphological changes of LDs in adipocytes during catecholamine-induced lipolysis. Shrinking of LDs occurred in some but not all adipocytes. Immunofluorescence imaging revealed that localization of phosphorylated HSL on the LD surface is necessary for LD shrinking. Catecholamine stimulation also induced development of small LDs in the periphery of almost all the cells. Because of shrinking of existing LDs and development of small LDs, adipocytes stimulated with catecholamine exhibit apparent LD fragmentation and dispersion.

5. Acknowledgements

We are grateful to Dr. Naohito Aoki (Mie Univ., Japan) and Dr. Teruo Kawada (Kyoto Univ., Japan) for helpful suggestions. This research was supported in part by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (20240023) to K.G.

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7. Figure legends

Fig. 1.

Time-lapse differential interference contrast (DIC) micrographs of differentiated adipocytes during catecholamine-induced lipolysis. The number in each image indicates the elapsed time (h) after 1×10^{-7} M norepinephrine administration. Existing lipid droplets (LDs) rapidly shrank in some adipocytes (*arrows*), whereas LDs in the other adipocytes were preserved or enlarged (*arrowheads*). Numerous small LDs (~1 μm) gradually appeared in almost all the cells, including undifferentiated ones, in the latter half of the observation period. Bar, 50 μm.

Fig. 2.

Catecholamine-induced activation and localization of hormone-sensitive lipase (HSL) on LD surface. The left and right columns show immunofluorescence micrographs of phosphorylated HSL (A, C, E, and G) and the corresponding DIC micrographs (B, D, F, and H), respectively.

Immunofluorescence staining was performed in adipocytes stimulated without (A and B) or with 1×10^{-7} M norepinephrine stimulation for 15 min (C and D), 2 h (E and F), and 8 h (G and H). HSL phosphorylated at serine 563 was strongly localized on the LD surface in some of the adipocytes stimulated with norepinephrine for 15 min or 2 h; in contrast, it was only slightly and hardly detected in adipocytes stimulated without or with norepinephrine for 8 h. Bar, 20 μm.

Fig. 3.

Catecholamine stimulation has no apparent effect on expression or distribution of adipose triglyceride lipase (ATGL). The left and right columns show immunofluorescence micrographs of ATGL (A and C) and the corresponding DIC micrographs (B and D), respectively. Immunofluorescence staining was performed in adipocytes stimulated without (A and B) or with 1×10^{-7} M norepinephrine stimulation for 2 h (C and D). ATGL shows a punctate distribution throughout the cytoplasm rather than on the LD surface, regardless of with or without norepinephrine stimulation. Bar, 20 μm.

Fig. 4.

Relationship between LD behavior and localization of phosphorylated HSL in four representative adipocytes. The left four columns show time-lapse DIC micrographs. The elapsed time (min) after 1×10^{-7} M norepinephrine administration is indicated in each column. The right column shows immunofluorescence micrographs of phosphorylated HSL. Immunofluorescence

staining was performed immediately after time-lapse observation for 120 min; thus, these immunofluorescence micrographs correspond to the DIC micrographs at 120 min. (A) Existing LDs shrank (*arrowheads*) and small LDs developed (*arrows*); phosphorylated HSL was localized on the LD surface. (B) Existing LDs shrank (*arrowhead*) but small LDs hardly developed; phosphorylated HSL was localized on the LD surface. (C) Existing LDs were preserved and small LDs developed (*arrows*); phosphorylated HSL was localized on the LD surface. (D) Existing LDs were preserved and small LDs developed (*arrows*); phosphorylated HSL was hardly detected. Bar, 10 μm.

8. Figures

Figure 1

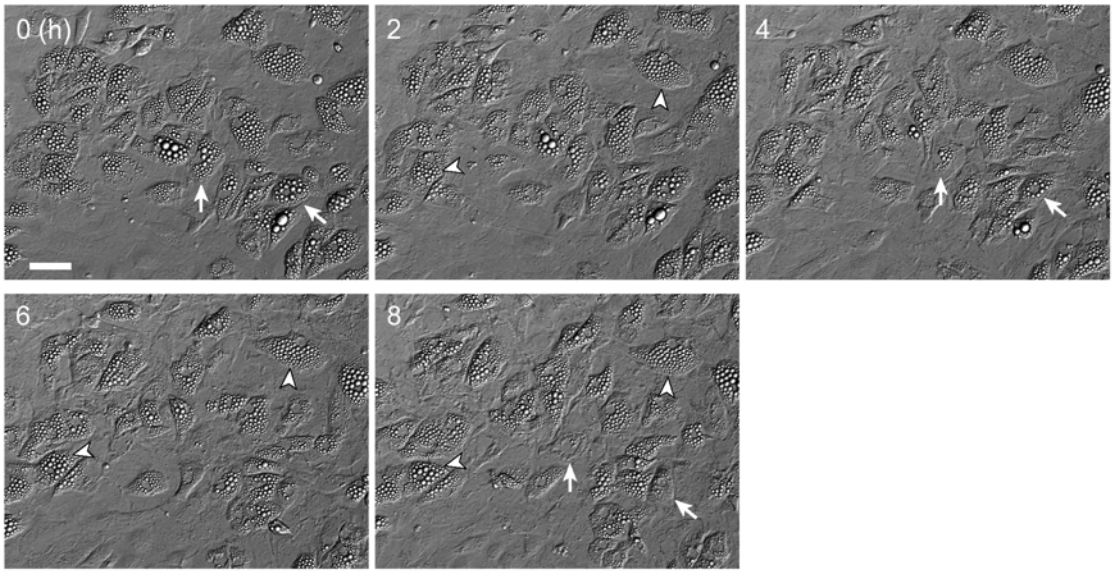


Figure 2

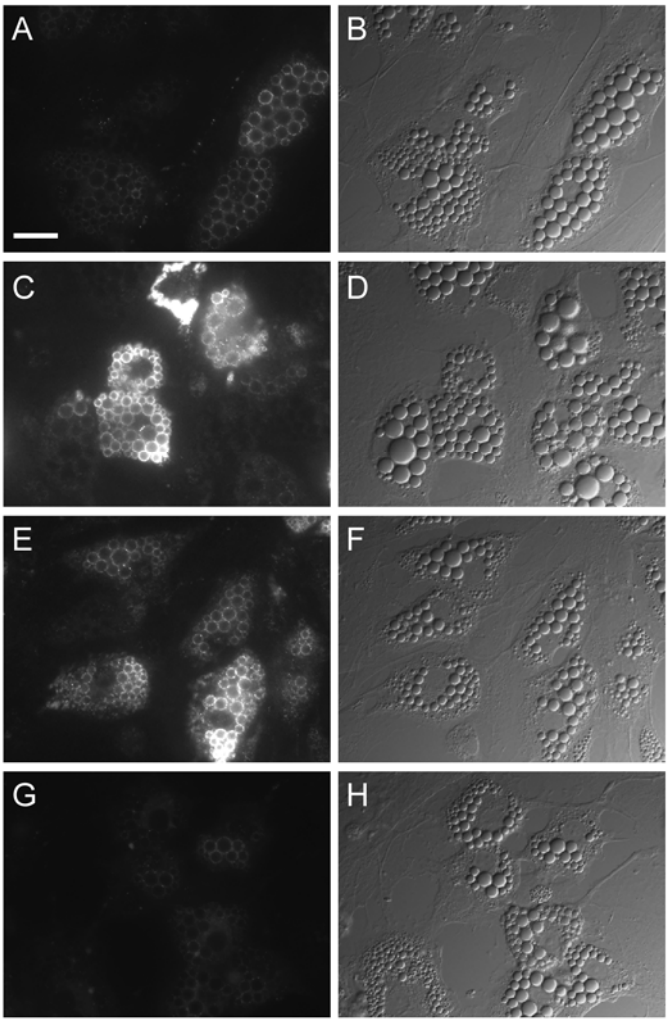


Figure 3

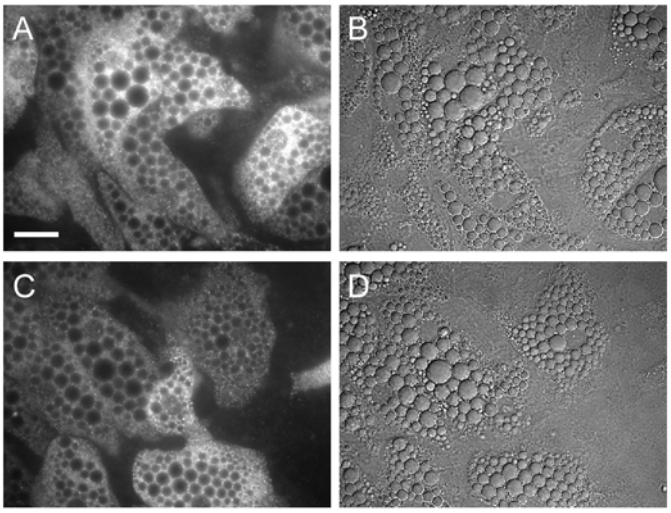
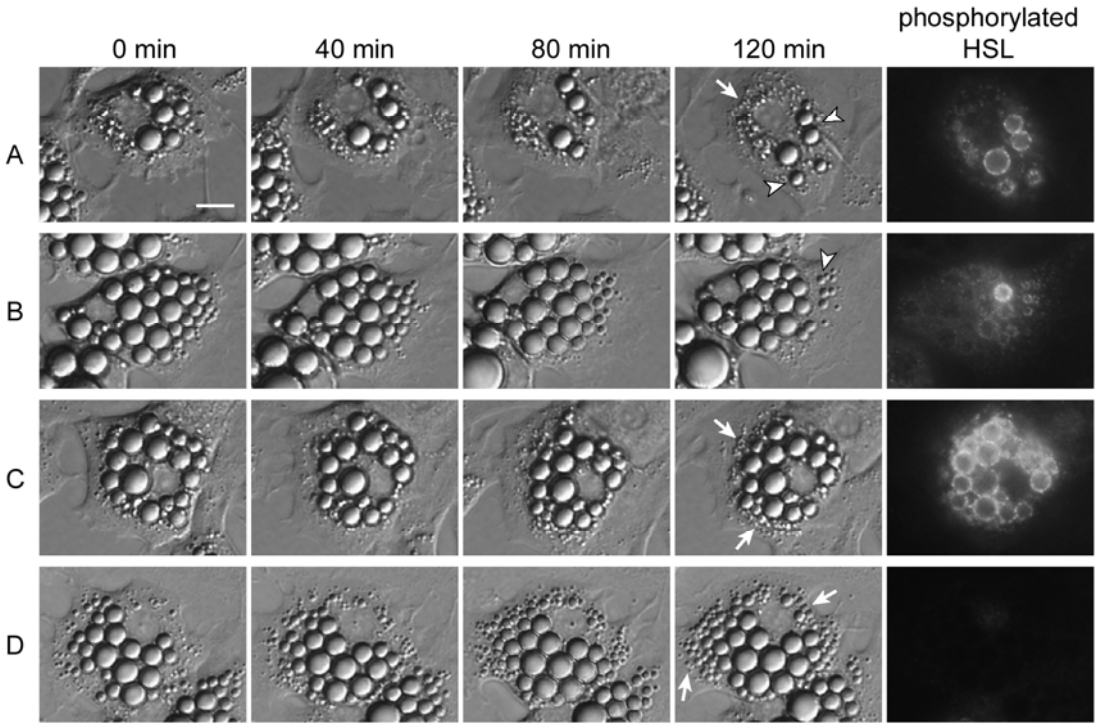


Figure 4



Supplementary data

Movie 1.

Time-lapse movie of adipocytes stimulated with 1×10^{-7} M norepinephrine. Movie duration is 8 hours (time lapse: 1 frame per minute) and corresponds to Fig. 1.

Movie 2.

Time-lapse movie of adipocytes stimulated with 1×10^{-7} M isoproterenol. The movie starts immediately after isoproterenol administration. Movie duration is 8 hours (time lapse: 1 frame per minute).

Movie 3.

Time-lapse movie of adipocytes stimulated with 1×10^{-7} M norepinephrine. The two adipocytes shown in Figs. 4A and 4C are seen in the movie. Movie duration is 120 minutes (time lapse: 1 frame per 30 seconds).

Movie 4.

Time-lapse movie of adipocytes stimulated with 1×10^{-7} M norepinephrine. The two adipocytes shown in Figs. 4B and 4D are seen in the movie. Movie duration is 120 minutes (time lapse: 1 frame per 30 seconds).