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Role of intracellular calcium in human adipocyte differentiation

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Shi, Hang, Yuan-Di Halvorsen, Pamela N. Ellis, William O. Wilkison, and Michael B. Zemel. Role of intracellular calcium in human adipocyte differentiation. *Physiol Genomics* 3: 75–82, 2000.—Intracellular calcium ($[Ca^{2+}]_i$) modulates adipocyte lipid metabolism and inhibits the early stages of murine adipogenesis. Consequently, we evaluated effects of increasing $[Ca^{2+}]_i$ in early and late stages of human adipocyte differentiation. Increasing $[Ca^{2+}]_i$ with either thapsigargin or A23187 at 0–1 h of differentiation markedly suppressed differentiation, with a 40–70% decrease in triglyceride accumulation and glycerol-3 phosphate dehydrogenase (GPDH) activity ($P < 0.005$). However, a 1-h pulse of either agent at 47–48 h only modestly inhibited differentiation. Sustained, mild stimulation of Ca^{2+} influx with either agouti protein or 10 mM KCl-induced depolarization during 0–48 h of differentiation inhibited triglyceride accumulation and GPDH activity by 20–70% ($P < 0.05$) and markedly suppressed peroxisome proliferator-activated receptor gamma (PPARγ) expression. These effects were reversed by Ca^{2+} channel antagonism. In contrast, Ca^{2+} pulses late in differentiation (71–72 h or 48–72 h) markedly increased these markers of differentiation. Thus increasing $[Ca^{2+}]_i$ appears to exert a biphasic regulatory role in human adipocyte differentiation, inhibiting the early stages while promoting the late stage of differentiation and lipid filling.

preadipocyte differentiation; intracellular calcium; obesity

INTRACELLULAR CALCIUM ($[Ca^{2+}]_i$) plays a key role in metabolic derangements associated with obesity (2, 7, 8). Increasing $[Ca^{2+}]_i$ via stimulation of either receptor or voltage-mediated calcium channels has also been shown to stimulate the expression and activity of fatty acid synthase (FAS), a key enzyme in de novo lipogenesis, and inhibit basal and agonist-stimulated lipolysis in both human and murine adipocytes (17, 38). These effects can be reversed by calcium channel antagonism (17, 38). Therefore, increasing $[Ca^{2+}]_i$ appears to promote triglyceride accumulation in adipocytes by exerting a coordinated control over lipogenesis and lipolysis, serving to simultaneously stimulate the former and suppress the latter, resulting in lipid filling and adipocyte hypertrophy.

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$[Ca^{2+}]_i$ has also been implicated in regulating adipogenesis, which has been thought to contribute to human and murine obesity (32). Ntambi and Takova (24) have reported that increasing $[Ca^{2+}]_i$, by either inhibiting Ca^{2+}-ATPase or stimulating Ca^{2+} influx, inhibited the early stages of murine adipocyte differentiation. However, the role of $[Ca^{2+}]_i$ in human adipocyte differentiation is unknown and may not be inferred from the rodent model, as cells derived from different species and development stages may show distinct patterns of responsiveness to various differentiation-inducing agents. In addition, the time course effects of $[Ca^{2+}]_i$ in early vs. late stages of differentiation is not well defined. Moreover, further investigation may assist in elucidating the role transition of $[Ca^{2+}]_i$ from preadipocyte, where it exerts a inhibitory effect in murine adipocyte differentiation, to mature adipocytes, where it acts as a lipogenic and antilipolytic signaling factor in regulating adipocyte metabolism.

Accordingly, the present study was designed to determine the effect of increasing $[Ca^{2+}]_i$ in both early and late stages of human adipocyte differentiation. We report here that increasing $[Ca^{2+}]_i$ in early stages of differentiation suppressed human adipocyte differentiation, similar to previous reports in the murine adipocyte cell line (8). In contrast, increasing $[Ca^{2+}]_i$ in late stages of differentiation promoted human adipocyte differentiation. Consequently, our data suggest that increasing $[Ca^{2+}]_i$ exerts a biphasic regulatory role in human adipocyte differentiation, serving to inhibit the early stages of differentiation, while promoting the late stages of differentiation and lipid filling.

MATERIALS AND METHODS

Culture and differentiation of human preadipocytes. Human preadipocytes used in this study were supplied by Zen-Bio (Research Triangle, NC). These preadipocytes originated from normal human subcutaneous adipose tissue and were isolated using a collagenase digestion and centrifugation method as previously described (13, 23). Preadipocytes were inoculated in DMEM/Ham's F-10 medium (DMEM-F10) (1:1, vol/vol) containing 10% FBS, 15 mM HEPES, and antibiotics at a density of 30,000 cells/cm^2. Confluent monolayers of preadipocytes were induced to differentiate with a standard differentiation medium consisting of DMEM-F10 (1:1, vol/vol) medium supplemented with 15 mM HEPES, 3% FBS, 33 μM biotin, 17 μM pantothenate, 100 nM insulin, 0.25 μM methylisobutylxanthine (MIX), 1 μM dexamethasone, 1 μM

BRL49653, and antibiotics with or without calcium agonists (thapsigargin and A23187). Preadipocytes were maintained in this differentiation medium for 3 days and subsequently cultured in adipocyte medium in which BRL49653 and MIX were omitted. Cultures were refed every 2–3 days.

$[Ca^{2+}]_i$ measurement. $[Ca^{2+}]_i$ in human preadipocytes was measured using a fura-2 dual-wavelength fluorescence imaging system. Preadipocytes were plated in 35-mm dishes with glass coverslips (P35G-0-14-C, MatTek). Prior to $[Ca^{2+}]_i$ measurement, cells were preincubated in serum-free medium overnight and rinsed with HEPES balanced salt solution (HBSS) containing the following components (in mM): 138 NaCl, 1.8 $CaCl_2$, 0.8 $MgSO_4$, 0.9 NaH_2PO_4, 4 $NaHCO_3$, 5 glucose, 6 glutamine, 20 HEPES, and 1% bovine serum albumin. Cells were loaded with fura-2 acetoxymethyl ester (fura-2 AM) (10 μM) in the same buffer for 2 h at 37°C in a dark incubator with 5% CO_2. To remove extracellular dye, cells were rinsed with HBSS three times and then postincubated at room temperature for an additional 1 h for complete hydrolysis of cytoplasmic fura-2 AM. The dishes with dye-loaded cells were mounted on the stage of Nikon TMS-F fluorescence inverted microscope with a Cohu model 4915 charge-coupled device (CCD) camera. Fluorescent images were captured alternatively at excitation wavelength of 340 and 380 nm with an emission wavelength of 520 nm. After establishment of a stable image baseline, the response to calcium agonists, thapsigargin and A23187, was determined. $[Ca^{2+}]_i$ was calculated using a ratio equation as described previously(12). Each analysis evaluated responses of 8–10 representative whole cells. Images were analyzed with InCyt Im2 version 4.62 imaging software (Intracellular Imaging, Cincinnati, OH). Images were calibrated using a fura-2 calcium imaging calibration kit (Molecular Probes, Eugene, OR) to create a calibration curve in solution, and cellular calibration was accomplished using digitonin (25 μM) and pH 8.7 Tris-EGTA (100 mM) to measure maximal and minimal $[Ca^{2+}]_i$ levels (12).

Triglyceride content assay. Human preadipocytes were incubated with calcium agonists as indicated during differentiation. Cellular triglyceride content was determined spectrophotometrically using a triglyceride assay kit (Sigma, St. Louis, MN). Cells were rinsed with Hanks' balanced salt solution and scraped in 0.9% saline. Cell suspension was then homogenized with sonication and subject to measurement.

GPDH activity assay. Glycerol-3-phosphate dehydrogenase (GPDH) activity was measured by a spectrophotometric method (18). Cells were scraped and sonicated in 250 mM sucrose solution containing 1 mM EDTA, 1 mM dithiothreitol, and 100 μM phenylmethylsulfonyl fluoride (pH 7.4). Homogenate was centrifuged at 18,500 *g* for 1 h, and the infranatant between the precipitate and the floating lipid layer was used for measuring oxidation rate of NADH.

Protein assay. Total cellular protein content for correction was measured by a modified Bradford method using Coomassie blue dye (Pierce, Rockford, IL).

Northern blot analysis. Northern blot analysis of peroxisome proliferator-activated receptor gamma (PPARγ), a critical transcriptional factor in adipocyte differentiation, was conducted as previously described (29). Total RNA from human adipocytes was extracted using $CsCl_2$ density centrifugation, run in 1% agarose gel, and transferred to nylon membrane (New England Nuclear, Boston, MA). The membrane was hybridized with a PPARγ cDNA probe radiolabeled using a random primer method. Unbound probe was removed by rinsing the membrane with 2× SSC/0.1% SDS for 30 min at room temperature and 0.1× SSC/0.1% SDS for 45 min at 55°C. Finally, the membrane was exposed to X-ray film (New England Nuclear, Boston, MA) at −80°C. All membranes were stripped and reprobed with 18S as loading control.

Statistical analysis. Replicates of 16 independent experiments were conducted for all triglyceride assays, 8 independent studies for GPDH activity, 10 independent experiments for the $[Ca^{2+}]_i$ measurements, and three independent experiments for the PPARγ mRNA measurements. All data are expressed as means ± SE and evaluated for statistical significance by one-way ANOVA using SPSS (SPSS, Chicago, IL).

RESULTS

To investigate the role of $[Ca^{2+}]_i$ in human adipocyte differentiation, we first selected thapsigargin, a Ca^{2+}-ATPase inhibitor, and A23187, a calcium ionophore, as calcium agonists to stimulate $[Ca^{2+}]_i$. Both agents are widely used to mimic physiological $[Ca^{2+}]_i$ mobilization in many cell types (1, 3, 6, 9). In this study, we demonstrate that thapsigargin and A23187 also stimulated $[Ca^{2+}]_i$ in human preadipocytes. A fura-2 dual-wavelength imaging system was used to measure $[Ca^{2+}]_i$ stimulation caused by calcium agonists. Figure 1, *top*, shows the cell fluorescence image in pseudocolor, which changed from green in baseline to yellow upon addition of 30 nM thapsigargin, indicating an increase of $[Ca^{2+}]_i$. Quantitation of this response demonstrated a threefold increase in $[Ca^{2+}]_i$ (baseline of 108 ± 12 nM vs. stimulated value of 456 ± 23 nM, $P < 0.001$). Similar results were observed in preadipocytes treated with 2 μM A23187 (baseline of 112 ± 14 nM vs. stimulated value of 412 ± 28 nM, $P < 0.001$), as shown in Fig. 1, *middle*. Similarly, using KCl as a depolarizing agent caused a slow, sustained increase in $[Ca^{2+}]_i$ (baseline of 117 ± 15 nM vs. stimulated value of 280 ± 26 nM, $P < 0.001$; Fig. 1, *bottom*).

To evaluate the effect of increasing $[Ca^{2+}]_i$ in early stages of differentiation, we treated human preadipocytes with calcium agonists during the first 48 h of differentiation, using triglyceride content and GPDH activity as late differentiation markers. Figure 2 illustrates that a 1-h pulse with 30 nM thapsigargin or 2 μM A23187 between the 23rd and 24th hour of differentiation suppressed subsequent triglyceride accumulation by 60 and 40% ($P < 0.005$), respectively. Similarly, a 1-h treatment with 30 nM thapsigargin or 2 μM A23187 at 23–24 h inhibited GPDH activity by 70 and 65% ($P < 0.005$, Fig. 3), respectively. However, a later 1-h pulse of thapsigargin or A23187 at 47–48 h of differentiation caused an inhibition in triglyceride content by only 20% ($P < 0.05$, Fig. 4), indicating an attenuation of their inhibitory effects on differentiation.

We next utilized more physiological $[Ca^{2+}]_i$ agonists, such as KCl, a cell membrane depolarization agent, and agouti protein, which is expressed in human adipose tissue and increases $[Ca^{2+}]_i$ in several cell types (19, 40), to stimulate Ca^{2+} influx during differentiation. Agouti protein was obtained as previously described (38). Figure 5 shows that treatment with 10 mM KCl or 100 nM agouti protein during 0–48 h of

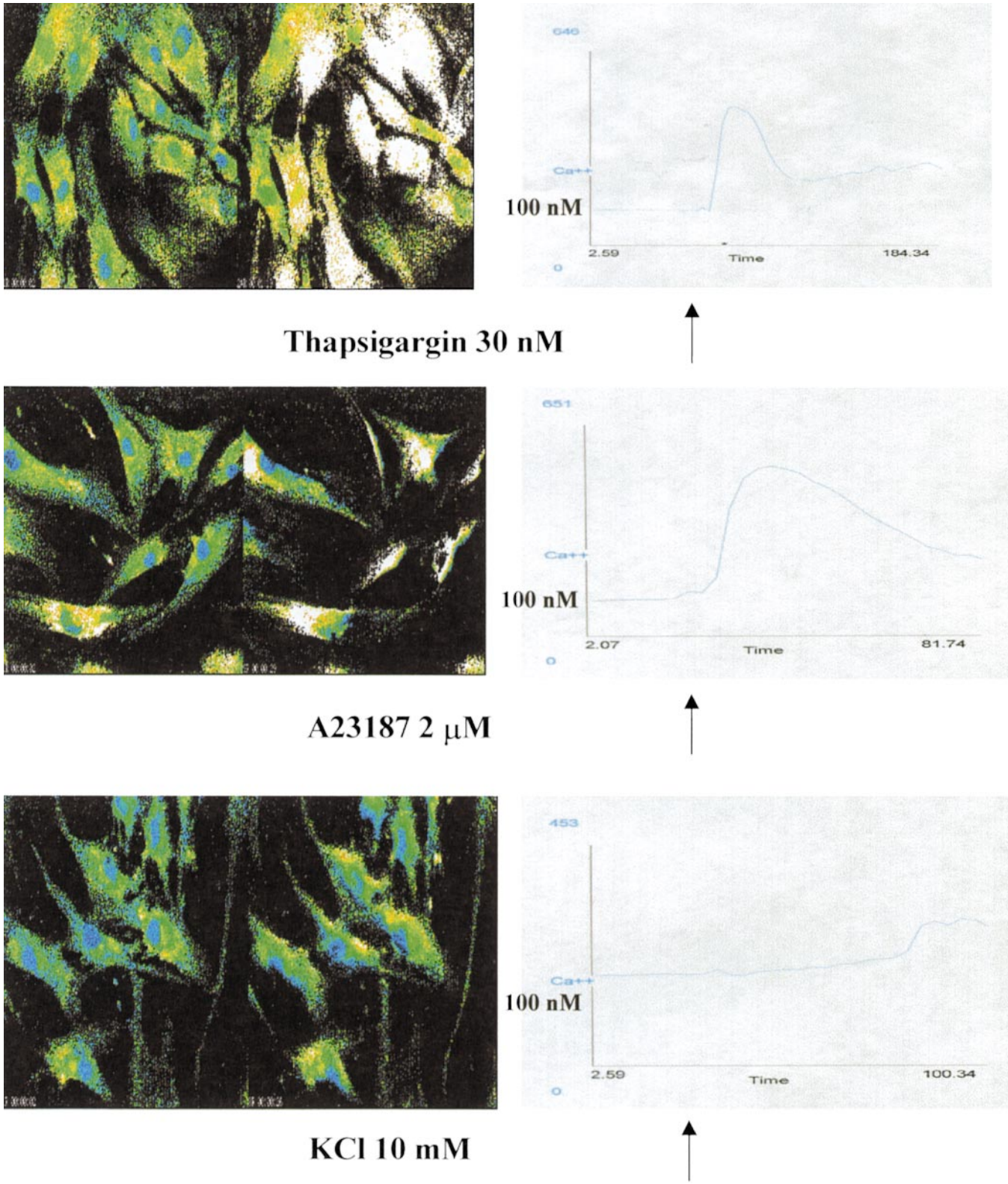

Fig. 1. The effects of thapsigargin, A23187, and KCl on human preadipocyte intracellular calcium ($[Ca^{2+}]_i$). A fura-2 dual-wavelength fluorescence imaging system was used to measure $[Ca^{2+}]_i$ as described in MATERIALS AND METHODS. Ten independent experiments were conducted for each treatment. *Left*: cell fluorescence images in pseudocolor, which change from green in baseline (*right*) to yellow (*left*) upon addition of each agonist, indicating increased $[Ca^{2+}]_i$. Quantitation of this response is shown in the graph on the *right*. The arrows indicate the time of addition of each agonist, as follows. *Top*: a 3-fold increase in $[Ca^{2+}]_i$ in response to 30 nM thapsigargin (456 ± 23 vs. 108 ± 12 nM $[Ca^{2+}]_i$, $P < 0.001$). *Middle*: similar results with 2 μM A23187 (412 ± 28 vs. 112 ± 14 nM $[Ca^{2+}]_i$, $P < 0.001$). *Bottom*: addition of 20 mM KCl caused a slow, sustained increase in $[Ca^{2+}]_i$ (280 ± 26 vs. 117 ± 15 nM $[Ca^{2+}]_i$, $P < 0.001$).

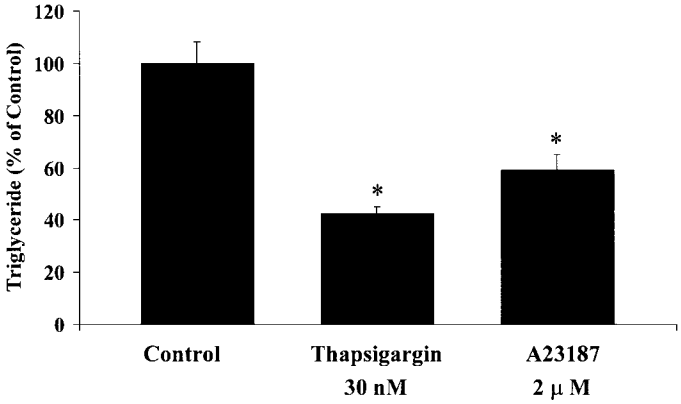

Fig. 2. The effects of the thapsigargin and A23187 treatment at 23–24 h of differentiation on human adipocyte triglyceride accumulation. Confluent monolayers of human preadipocytes were induced to differentiation with a standard adipocyte medium supplemented with or without thapsigargin or A23187 as indicated. A 1-h pulse treatment of human preadipocytes with 30 nM thapsigargin or 2 μM A23187 was conducted at 23–24 h of differentiation. Total cellular triglyceride content was measured at the end of *day 8* of differentiation as described in MATERIALS AND METHODS. Data are normalized to percent of control (0.988 ± 0.079 μg/μg protein). *$P < 0.005$ vs. control; $n = 16$.

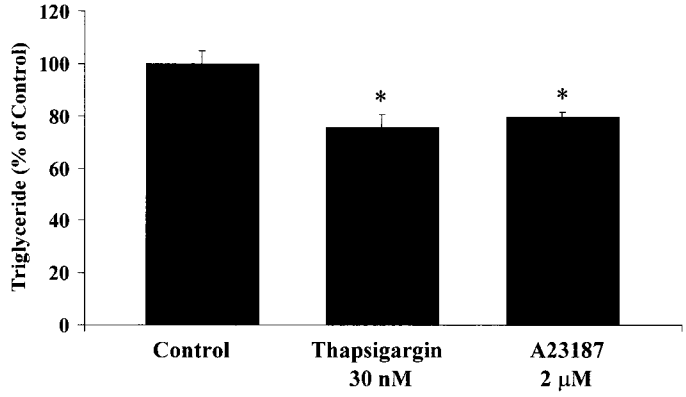

Fig. 4. The effects of the thapsigargin and A23187 treatment at 47–48 h of differentiation on human adipocyte triglyceride accumulation. Confluent monolayers of human preadipocytes were induced to differentiation with a standard adipocyte medium supplemented with or without thapsigargin or A23187 as indicated. A 1-h pulse treatment of human preadipocytes with 30 nM thapsigargin or 2 μM A23187 was conducted at 47–48 h of differentiation. Total cellular triglyceride content was measured at the end of *day 8* of differentiation as described in MATERIALS AND METHODS. Data are normalized to percent of control (1.04 ± 0.05 μg/μg protein). *$P < 0.005$ vs. control; $n = 16$.

differentiation suppressed triglyceride content by 70 and 20% ($P < 0.01$), respectively. Similarly, a long-term treatment with 10 mM KCl or 100 nM agouti protein during 0–48 h of differentiation caused an inhibition in GPDH activity by 40 and 20% ($P < 0.05$, Fig. 6), respectively. This inhibition was completely prevented by 30 nM nitrendipine, an L-type Ca^{2+} channel antagonist. Moreover, using PPARγ expression as a differentiation marker, we demonstrated that long-term treatment with 10 mM KCl or 100 nM agouti during 0–48 h of differentiation greatly inhibited PPARγ expression, which was totally recovered by nitrendipine (Fig. 7).

We next assessed the effect of increasing $[Ca^{2+}]_i$ in late stages of differentiation. In contrast to early stage of treatment, a 1-h pulse treatment with 30 nM thapsigargin or 2 μM A23187 at 71–72 h of differentiation increased triglyceride content by 90 and 65% ($P < 0.001$, Fig. 8), respectively. Similarly, Fig. 9 illustrates that a sustained treatment with 10 mM KCl or 100 nM agouti protein during 48–72 h of differentiation enhanced triglyceride accumulation by two- to threefold ($P < 0.001$). Furthermore, Fig. 10 shows that a long-term treatment with either KCl or agouti protein late

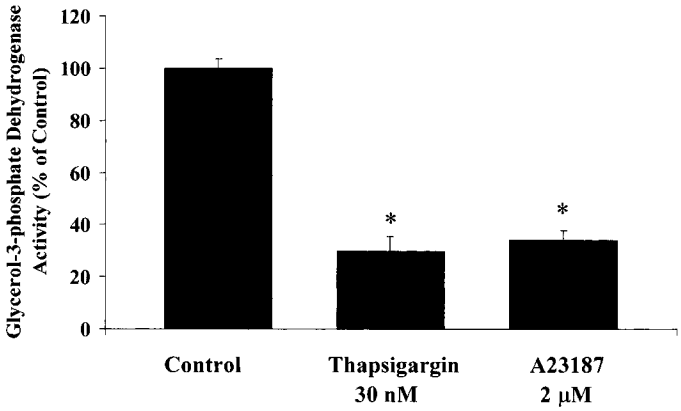

Fig. 3. The effects of the thapsigargin and A23187 treatment at 23–24 h of differentiation on human adipocyte glycerol-3-phosphate dehydrogenase (GPDH) activity. Confluent monolayers of human preadipocytes were induced to differentiation with a standard adipocyte medium in the presence or absence of thapsigargin or A23187 as indicated. A 1-h pulse treatment of human preadipocytes with 30 nM thapsigargin or 2 μM A23187 was conducted at 23–24 h of differentiation. GPDH activity was measured at the end of *day 8* of differentiation as described in MATERIALS AND METHODS. Data are normalized to percent of control oxidation of NADH (23.2 ± 0.9 $nmol \cdot min^{-1} \cdot mg\ protein^{-1}$). *$P < 0.005$ vs. control; $n = 8$.

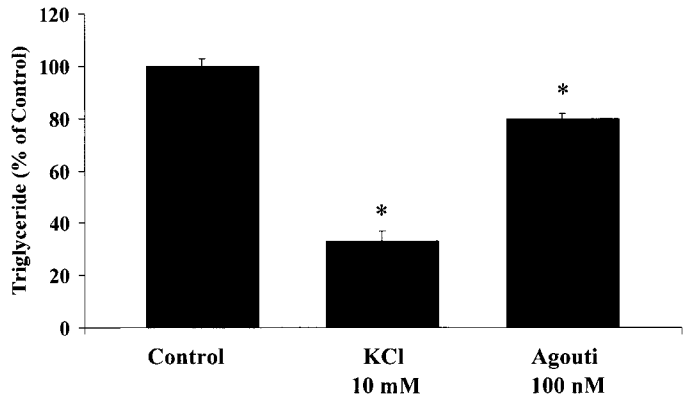

Fig. 5. The effects of KCl and agouti protein treatment during 0–48 h of differentiation on human triglyceride accumulation. Confluent monolayers of human preadipocytes were induced to differentiation with a standard adipocyte medium in the presence or absence of KCl or agouti as indicated. KCl and agouti protein were removed after 2-day treatment during 0–48 h of differentiation. Total cellular triglyceride content was measured at the end of *day 8* of differentiation as described in MATERIALS AND METHODS. Data are normalized to percent of control (1.46 ± 0.12 μg/μg protein). *$P < 0.01$ vs. control; $n = 16$.

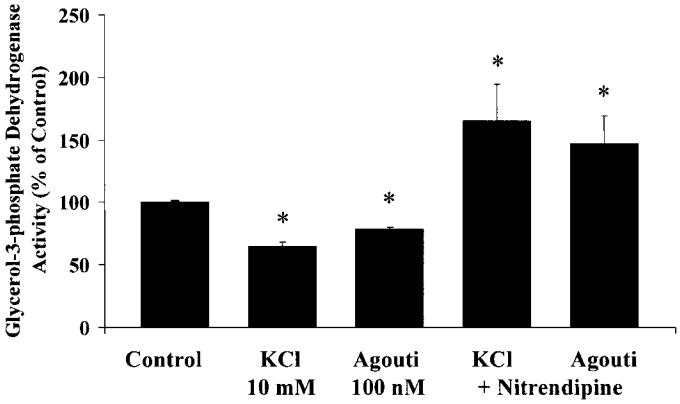

Fig. 6. The effects of KCl and agouti protein treatment during 0–48 h of differentiation on human GPDH activity. Confluent monolayers of human preadipocytes were induced to differentiation with a standard adipocyte medium in the presence or absence of KCl or agouti alone, or KCl or agouti plus nitrendipine as indicated. KCl and agouti protein were removed after 2-day treatment during 0–48 h of differentiation. GPDH activity was measured at the end of *day 8* of differentiation as described in MATERIALS AND METHODS. Data are normalized to percent of control NADH oxidation (20.4 ± 0.2 nmol·min^{-1}·mg protein^{-1}). *$P < 0.05$ vs. control; $n = 8$.

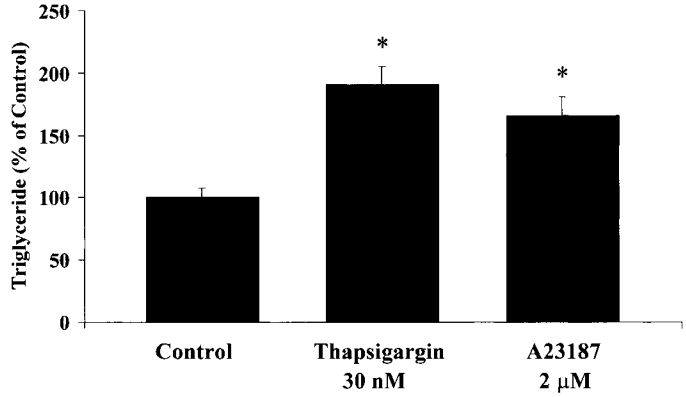

Fig. 8. The effects of the thapsigargin and A23187 treatment at 71–72 h of differentiation on human adipocyte triglyceride accumulation. Confluent monolayers of human preadipocytes were induced to differentiation with a standard adipocyte medium supplemented with or without thapsigargin or A23187 as indicated. A 1-h pulse treatment of human preadipocytes with 30 nM thapsigargin or 2 μM A23187 was conducted at 71–72 h of differentiation. Total cellular triglyceride content was measured at the end of *day 8* of differentiation as described in MATERIALS AND METHODS. Data are normalized to percent of control (0.93 ± 0.07 μg/μg protein). *$P < 0.001$ vs. control; $n = 16$.

in differentiation caused a marked increase in PPARγ expression.

DISCUSSION

$[Ca^{2+}]_i$ appears to play a key role in metabolic disorders associated with obesity, and sustained high levels of $[Ca^{2+}]_i$ may contribute to this derangement (2, 7, 8). In this study, we demonstrate that $[Ca^{2+}]_i$ plays a regulatory role in adipogenesis, an important contributor to increased fat tissue mass. Our data suggest that increasing $[Ca^{2+}]_i$ in the early stages of differentiation inhibits human adipocyte differentiation, whereas increasing $[Ca^{2+}]_i$ in late stage promotes human adipocyte differentiation. This delineates a role transition in $[Ca^{2+}]_i$, which serves to inhibit differentiation in the early stages but promote differentiation in late stages.

$[Ca^{2+}]_i$ appears to promote and accelerate preadipocyte differentiation program(s), thereby inducing the adipocyte phenotype in the late stage of differentiation. To achieve this, increasing $[Ca^{2+}]_i$ causes a marked

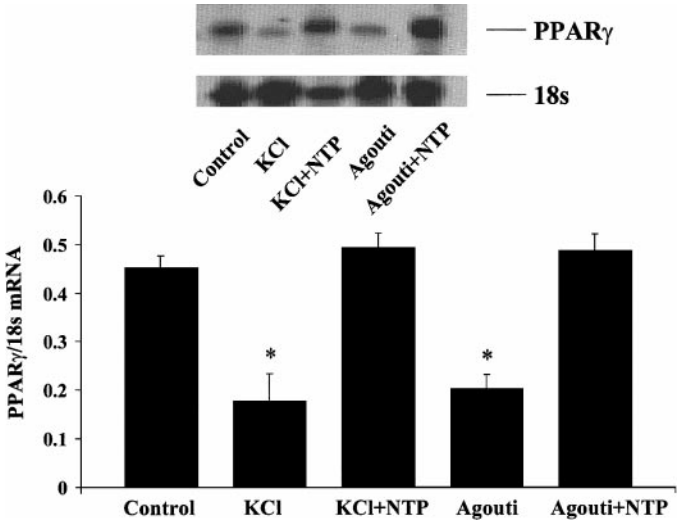

Fig. 7. The effects of KCl and agouti protein treatment during 0–48 h of differentiation on human preadipocyte PPARγ expression. Confluent monolayers of human preadipocytes were induced to differentiation with a standard adipocyte medium in the presence or absence of KCl or agouti alone, or KCl or agouti plus nitrendipine (+NTP) as indicated. KCl and agouti protein were removed after 2-day treatment during 0–48 h of differentiation. Total RNA was prepared at the end of *day 4*. Northern blot analysis was performed as described in MATERIALS AND METHODS. PPARγ, peroxisome proliferator-activated receptor gamma. Blot shown is representative of 3 similar experiments. *$P < 0.05$.

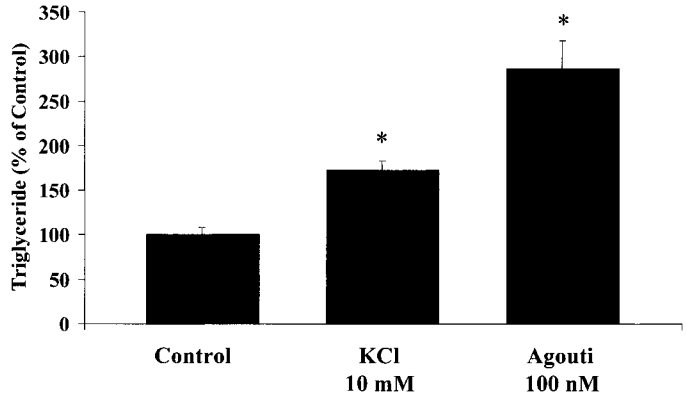

Fig. 9. The effects of KCl and agouti protein treatment during 48–72 h of differentiation on human triglyceride accumulation. Confluent monolayers of human preadipocytes were induced to differentiation with a standard adipocyte medium in the presence or absence of KCl or agouti as indicated. A 1-day treatment of human preadipocytes with 10 mM KCl or 100 nM agouti was conducted during 48–72 h of differentiation. Total cellular triglyceride content was measured at the end of *day 8* of differentiation as described in MATERIALS AND METHODS. Data are normalized to percent of control (1.46 ± 0.12 μg/μg protein). *$P < 0.01$ vs. control; $n = 16$.

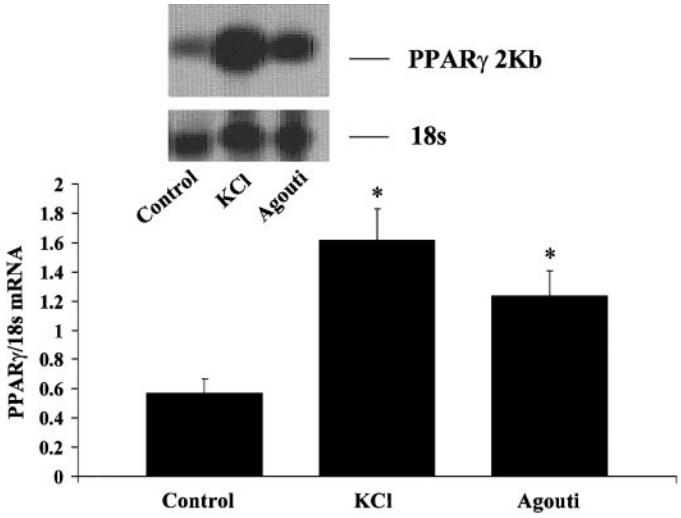

Fig. 10. The effects of KCl and agouti protein treatment during 48–72 h of differentiation on human preadipocyte PPARγ expression. Confluent monolayers of human preadipocytes were induced to differentiation with a standard adipocyte medium in the presence or absence of KCl or agouti as indicated. A 1-day treatment of human preadipocytes with 10 mM KCl or 100 nM agouti was performed during 48–72 h of differentiation. Total RNA was prepared at the end of *day 4*. Northern blot analysis was conducted as described in MATERIALS AND METHODS. Blot shown is representative of three similar experiments. $^*P < 0.05$.

increase in the expression of PPARγ (Fig. 10), a nuclear hormone receptor that acts as a critical transcriptional factor in adipocyte differentiation programs (31). Increased expression of PPARγ may subsequently accelerate adipocyte differentiation by directly acting upon and eliciting late differentiation gene expression, such as aP2, steroyl-CoA desaturase (SCD-1), phospho*enol*-pyruvate carboxykinase (PEPCK), and FAS (11, 16, 33–36). On the other hand, in late differentiation, preadipocytes become more committed to terminal differentiation after the expression of several critical differentiation transcriptional factors, such as CCAAT/enhancer binding protein beta and delta (C/EBPβ and C/EBPδ), PPARγ, and sterol regulatory element binding protein-1/adipocyte determination and differentiation factor-1 (SREBP-1/ADD-1) (5, 11, 22, 31). $[Ca^{2+}]_i$ may synergize with these transcriptional factors to promote the differentiation program by stimulating late differentiation gene expression. Consistent with this, $[Ca^{2+}]_i$ acts as a lipogenic and antilipolytic signaling factor in regulating adipocyte metabolism in both rodent and human adipocytes (17, 38). Previous reports from our laboratory demonstrate that increasing $[Ca^{2+}]_i$ stimulates the expression and activity of FAS, a key enzyme in de novo lipogenesis and thereby increases triglyceride storage (17). In addition, we have also shown that increasing $[Ca^{2+}]_i$ inhibits basal and agonist-stimulated lipolysis in primary cultured human adipocytes (38). These effects can be completely blocked by calcium channel antagonism (17, 38). Therefore, increasing $[Ca^{2+}]_i$ appears to promote triglyceride accumulation in adipocytes by exerting a coordinated control over lipogenesis and lipolysis, serving to simultaneously stimulate the former and suppress the latter, thereby resulting in lipid filling and adipocyte hypertrophy. Accordingly, the lipogenic and anti-lipolytic effects of $[Ca^{2+}]_i$, coupled with increased expression of a differentiation transcriptional factor such as PPARγ, may contribute to the stimulatory effect of $[Ca^{2+}]_i$ in the late stages of differentiation.

However, data from Ntambi and Takova (24) did not show the stimulatory effect of increasing $[Ca^{2+}]_i$ in late stages of differentiation in 3T3-L1 cells. The reason for this discrepancy is not clear. A possible explanation is that these two studies used two different cell models, 3T3-L1 derived from mouse embryo and human preadipocytes originating from human stromal-vascular cells in subcutaneous fat depot (11). Therefore, cell models derived from different species and development stage may exhibit distinct differentiation properties.

In contrast to the stimulatory effect of increasing $[Ca^{2+}]_i$ in late stages of differentiation, increasing $[Ca^{2+}]_i$ in the early stage suppresses human adipocyte differentiation. In agreement with our findings, increasing $[Ca^{2+}]_i$ has been shown to exhibit an inhibitory effect in early stages of murine adipocyte differentiation (24). $[Ca^{2+}]_i$ exerted this inhibitory effect by blocking the postconfluent mitotic phase and mediating sustained levels of *c-myc* expression (24). This sustained *c-myc* expression precludes cell entry to the G_D stage necessary for subsequent differentiation (27, 28, 37) and instead forces cells to re-enter the normal cell cycle (10). In this study, increasing $[Ca^{2+}]_i$ in early stage of differentiation caused a substantial inhibition in PPARγ expression (Fig. 7), which would be expected to subsequently reduce late differentiation gene expression and thereby inhibit further differentiation.

The mechanism whereby $[Ca^{2+}]_i$ undergoes this role transition during human adipocyte differentiation is unknown. However, cAMP also plays a discordant role in adipocyte differentiation and mature adipocyte metabolism. Increasing cAMP promotes adipocyte differentiation (10), whereas it inhibits expression and activity of FAS (25, 30), a key enzyme in de novo lipogenesis, and stimulates lipolysis in mature adipocytes. Moreover, there is a significant interaction between the calcium and cAMP signaling pathways. Structural and functional studies have demonstrated adenylyl cyclases are associated with the site of Ca^{2+} entry into the cell and that Ca^{2+} entry causes a marked inhibition of type V and VI adenylyl cyclases, thereby reducing cAMP levels in several cell types (4). Alternatively, we have recently demonstrated that increasing human adipocyte $[Ca^{2+}]_i$ stimulates phosphodiesterase 3B activity, resulting in reduced cAMP levels (39). Accordingly, increasing $[Ca^{2+}]_i$ in the early stages of differentiation may suppress pre-adipocyte cAMP levels and thereby inhibit differentiation. Conversely, a $[Ca^{2+}]_i$-induced decrease in cAMP late in differentiation upregulates lipogenesis and suppresses lipolysis, thereby promoting adipocyte maturation and lipid filling.

It was originally believed that only early-onset obesity was associated with adipocyte hyperplasia,

whereas maturity-onset obesity was believed to result solely from adipocyte hypertrophy (15, 26). This concept was challenged by later studies demonstrating that the potential to acquire new adipocytes persists even at the adult stage. Several lines of evidence demonstrate that an increase in fat cell number appears to be well correlated with severity of human obesity in adult life (14). Moreover, specific early differentiation genes have been reported to be expressed in adipose tissue from very old mice (20). In addition, fat cell precursors, such as stromal-vascular cells, isolated from adult human adipose tissue can be fully differentiated into mature adipocytes in vitro (13, 21). Furthermore, adipocyte development has been shown to depend on pre-adipocyte recruitment in vivo (21). Although the relative contribution of adipocyte hypertrophy vs. hyperplasia to human adiposity is unknown, the capacity to generate new adipocytes from cells persists throughout life span and clearly plays a role in regulating fat tissue mass. Our data in the present study implicate a role of $[Ca^{2+}]_i$ in modulating adipocyte hyperplasia and further affecting adipose tissue mass in vivo. The significance of $[Ca^{2+}]_i$ in regulating adipogenesis bears physiological basis, as there are many nutritional, hormonal, and pharmaceutical factors that can modulate $[Ca^{2+}]_i$ signaling in both adipocytes and preadipocytes. In addition to the $[Ca^{2+}]_i$ mobilization agents used in this study (cell membrane depolarization and agouti protein), other endocrine and/or paracrine factors, including 1,25-dihydroxyvitamin D, parathyroid hormone (41), angiotensin II, and arginine vasopressin (unpublished data), have also been demonstrated to mobilize adipocyte $[Ca^{2+}]_i$. Furthermore, we recently reported that sulfonylureas, a family of insulin secretagogues widely used to stimulate insulin release in the treatment of type II diabetes, stimulates $[Ca^{2+}]_i$ and thereby modulates lipid metabolism in human adipocytes (29). Thus $[Ca^{2+}]_i$ appears to exhibit a physiological role in regulating adipogenesis and fat tissue mass formation.

In summary, our data suggest that increasing $[Ca^{2+}]_i$ exerts a biphasic regulatory role in human adipocyte differentiation, serving to inhibit the early stages of differentiation while promoting the late stages of differentiation and lipid filling. We conclude that the role of $[Ca^{2+}]_i$ in adipocyte differentiation may not only provide insight into the mechanism of human adipogenesis and energy homeostasis but also represents an important target for further development of therapeutic intervention in obesity.

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