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Staurosporine Rapidly Commits 3T3-F442A Cells to the Formation of Adipocytes by Activation of GSK-3β and Mobilization of Calcium

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ABSTRACT

Pre-adipose 3T3-F442A cells exposed to fetal bovine serum or human growth hormone (adipogenic medium) become irreversibly committed to differentiation into adipocytes within 24–36 h. We show now that the action of the serine-threonine kinase inhibitor staurosporine is much more rapid since its addition in non-adipogenic medium resulted in commitment to adipocyte differentiation within 4–6 h. During this period, glycogen synthase kinase 3β was activated. Commitment depended on an increase in the intracellular calcium concentration that was modulated in part by a T-type calcium channel since mibefradil, amiloride, and $NiCl_2$, which are selective blockers of the T-type channels, partially inhibited adipose differentiation. Studies of the inhibitory action of retinoic acid showed that a period of time after exposure to St was required in order to stabilize the commitment to adipose differentiation. It was concluded that the commitment of the cells consists of two stages. Commitment is promoted during the first one, and during the second there is a stabilization which still can be destabilized by the addition of retinoic acid or other drugs. The commitment becomes stable after 40 h of staurosporine treatment, and can no longer be prevented by retinoic acid. The identification of these two stages of commitment makes it possible to analyze in further detail early molecular events of the process and the nature of any other participating genes. J. Cell. Biochem. 105: 147–157, 2008. © 2008 Wiley-Liss, Inc.

KEY WORDS: ADIPOSE CONVERSION; CELL COMMITMENT; GSK-3β; CALCIUM MOBILIZATION; STAUROSPORINE; LITHIUM CHLORIDE; RETINOIC ACID

The activation and/or inactivation of specific genes during an early period after cell interaction with inducers are essential for cell commitment to a differentiation pathway. The 3T3-F442A preadipocytes are a subclone of 3T3 Swiss mouse embryo fibroblasts [Green and Kehinde, 1976]. This is a well characterized preadipocyte cell line which differentiates into adipocytes in vitro [Green and Kehinde, 1976] or into fat pads when injected subcutaneously into nude mice [Green and Kehinde, 1979]. However, it has also been described that this cell line has the ability to differentiate into osteoblasts. BMP signaling and retinoic acid cooperate to repress adipocyte conversion, stimulate proliferation and induce their differentiation into fully developed osteoblasts with extracellular matrix calcification in culture [Skillington et al., 2002], which suggest that at least the 3T3-F442A cells can commit to either adipocytes or osteoblasts depending on their exposure to a specific inducer. Moreover, the 3T3-L1 preadipocytes, a sister cell line of the 3T3-F442A, undergo macrophage conversion after peritoneal injection in mice [Charriere et al., 2003]. These reports give support to the idea that both 3T3 sub-lines are not yet committed to differentiate only into adipose cells.

When the 3T3-F442A cells follow the adipocyte differentiation lineage, they have to reach the resting state under appropriate culture conditions and undergo adipose conversion through gene expression programmed changes [reviewed by MacDougald and Lane, 1995]. Adipose conversion has three major steps: commitment, clonal expansion of committed cells, and phenotype expression [Green et al., 1985; Pairault and Lasnier, 1987; Castro-Muñozledo et al., 2003]. Prostaglandin F2α [Russell and Ho, 1976], prostacyclin [Négrel et al., 1989], 1-methyl-3-isobutyl xanthine (MIX) [Russell and Ho, 1976], dexamethasone (DEX) [Rubin et al., 1978] or indomethacin [Williams and Polakis, 1977] accelerate the expression of the adipose phenotype. Insulin, IGF-1 and L-T3 increase clonal expansion and regulate the lipogenic enzyme activities of terminally differentiated adipocytes [Steinberg and Brownstein, 1982; Zezulak and Green, 1985; Flores-Delgado et al., 1987; Guller et al., 1988; Dani et al., 1989], whereas growth

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hormone (GH) [Morikawa et al., 1982, 1984; Nixon and Green, 1984] and serum adipogenic factors [Kuri-Harcuch and Green, 1978; Hauner and Löffler, 1986; Kuri-Harcuch, unpublished results], seem to be involved in promoting commitment [Castro-Muñozledo et al., 2003]. Interferon-γ [Keay and Grossberg, 1980], retinoids [Murray and Russell, 1980; Kuri-Harcuch, 1982; Castro-Muñozledo et al., 1987], TGF-β [Ignotz and Massague, 1985], and TNFα [Castro-Muñozledo et al., 2003] inhibit adipocyte differentiation. Nearly all work on adipogenesis using cultured cells has been carried out on 3T3-L1 or on 3T3-F442A cells. There are 3,000 studies and 570,000 citations on the first and 400 studies and 59,000 citations on the second in the literature. However, very few studies have examined the earliest stages of adipogenesis and particularly the process of commitment. These studies have only shown that commitment into adipocytes is inhibited with TGFβ, TNFα or retinoic acid, and that it seems to take place during a long period of about 40 h, after which clonal expansion and phenotype expression follow [Kuri-Harcuch, 1982; Ignotz and Massague, 1985; Castro-Muñozledo et al., 1987, 2003].

In what follows, we will concentrate on the poorly understood process of commitment. We previously showed that commitment to differentiation required 24–36 h of adipogenic stimulation with fetal bovine serum (FBS) or human growth hormone (hGH) [Castro-Muñozledo et al., 2003]. In this article, we show that, in the absence of any other adipogenic factors, staurosporine (St), a serine-threonine kinase inhibitor [Tamaoki et al., 1986], acts much more rapidly, within 4–6 h, in committing the cells to adipogenesis. The requirement of GSK3β activation and an increase in intracellular Ca^{2+}, mediated in part by T-type calcium channels, is also shown. By studying the effect of retinoic acid, we also determined that there is a period during which the commitment is unstable, as it can be destabilized by the action of RA.

MATERIALS AND METHODS

MATERIALS

Recombinant human growth hormone (RhGH) was from Genentech, Inc. (San Francisco, CA). Epidermal growth factor (EGF) was from IMCERA Bioproducts, Inc. (Terre Haute, IN). Fetal bovine serum (FBS) was from M.A. Bioproducts (Walkersville, MD), calf serum was from HyClone laboratories, Inc. (Logan, UT) and cat serum was obtained by bleeding adult domestic cats. Eagle's medium modified by Dulbecco-Vögt (DMEM) was purchased from Invitrogen-Life Technologies (Carlsbad, CA). Insulin, d-biotin, human transferrin, triiodothyronine, staurosporine, *all*-trans-retinoic acid (RA), Oil Red O, dimethyl sulfoxide (DMSO), amiloride, and bovine serum albumin (BSA) were from Sigma Chemical Co. (St. Louis, MO). Mibefradil was from Calbiochem-Novabiochem International (La Jolla, CA). Calcium orange-AM was purchased from Molecular Probes, Inc. (Eugene, OR). All other reagents were analytical grade.

CELL CULTURE

The 3T3-F442A preadipose cells [Green and Kehinde, 1976] were plated as described previously [Castro-Muñozledo et al., 2003]; 3 days after plating, cultures were fed with DMEM supplemented with 4% (v/v) cat serum, 5 μg/ml insulin, and 1 μM d-biotin (non-adipogenic medium) [Kuri-Harcuch and Green, 1978], and refed every other day. One-to-two day post-confluent cultures were stimulated to differentiate with DMEM containing 2%(v/v) cat serum, 0.2%(v/v) donor adult bovine serum, 5.0 μg/ml insulin, 5.0 μg/ml transferrin, 1.0 μM d-biotin, 2 nM triiodothyronine, 40 μM 2-mercaptoethanol and 0.01 ng/ml EGF (definitive non-adipogenic medium) [Morikawa et al., 1984], plus 11 nM staurosporine as indicated. After stimulation, cultures were rinsed twice with serum-free medium, changed to definitive non-adipogenic medium without adipogenic stimulus and maintained during 6 days without any further changes of medium. Treatment with the indicated drugs was carried out as described for each experiment; DMSO concentration in culture medium was never higher than 0.1% (v/v). For experiments in serum-free medium, preconfluent cultures were washed three times with serum-free DMEM, refed with DMEM-F12 (1:2, v/v) containing 1 mg/ml BSA, 10 μg/ml transferrin, 0.15 nM triiodothyronine, 50 ng/ml EGF, 5 μg/ml insulin and 1 μM d-biotin, and maintained for 7–10 days without any further changes of medium [Salazar-Olivo et al., 1995]. All cultures were incubated at 37°C under 10% CO_2 atmosphere. The results correspond to the average from, at least, three different duplicated experiments.

Adipose conversion was quantified by lipogenic enzyme activity, by lipid staining or by determining the number of adipocyte clusters in stained cultures. Intracellular lipids stained with Oil Red O were extracted and quantified from cell cultures as previously described [Ramírez-Zacarías et al., 1992]. Glycerophosphate dehydrogenase (GPDH) (EC 1.1.1.8.) activity was determined in cell extracts as previously described [Castro-Muñozledo et al., 1987]. Protein concentration was determined by the method of Lowry et al. [1951].

RT-PCR PRIMERS AND CONDITIONS

RNA was extracted from 3T3-F442A cells with Trizol (Invitrogen Co.). Then, 4 μg of total RNA was used for cDNA synthesis with Superscript II reverse transcriptase using oligo-dT (12–18) primers. One microliter of the cDNA reaction was used for PCR amplification. After an initial treatment for 4 min at 95°C, amplification was done in the presence of 0.2 μM of each primer as follows: 25 cycles, 95°C, 40 s; 66°C, 40 s; 72°C, 1 min; final extension was made at 72°C, 10 min. The primers used for PCR reactions were the following: for Calcium Channel T-type α1G (Accession number NM_009783), Cav 3.1-A (5′-CTCTCGCCGCACCAGTAGCAGT-3′) (forward) and Cav 3.1-B (5′-GGGGAGGGAAGATATAGGCAGACC-3′) (reverse) to amplify a Cav3.1 specific 593 bp fragment. As an internal control, the acidic ribosomal phosphoprotein PO (Accession number NM_007475) was amplified with primers PR PO-A (5′-AGGCCCTG-CACTCTCGCTTTCTGG-3′) (forward) and PR PO-B (5′-TGGTTG-CTTTGGCGGGATTAGTCG-3′) (reverse) in order to obtain a specific 345 bp fragment. The PCR products were analyzed in 1% (w/v) agarose gels.

IMMUNOLOCALIZATION OF Ca^{2+} CHANNEL PROTEIN IN –3T3-F442A CELLS

Immunostaining of the α1-G calcium channel subunit protein was carried-out with a polyclonal antibody purchased from Alomone Labs Ltd. (Jerusalem, Israel) (anti-Cav 3.1). Cultures were fixed with 3.5% (w/v) p-formaldehyde and they were washed twice with PBS.

Cells were permeabilized during 10 min with 0.005% (v/v) Triton X-100 in PBS, and then they were washed twice with PBS and blocked for 2 h with 10% calf serum in PBS (v/v). Cells were incubated with the primary antibody anti-Cav 3.1 (1:100 dilution) at 4°C overnight; cultures with differentiated adipocytes were incubated with the same dilution of primary antibody during 2 h in presence of 0.1% saponin. Then they were washed six times with 0.05% (v/v) Tween-20 in PBS and they were incubated with the FITC-conjugated secondary antibody (1:200 dilution; ICN) during 2 h at room temperature. At the end of incubation, cells were washed six times with 0.05% (v/v) Tween-20 in PBS and mounted with VECTASHIELD® Mounting Medium (Vector Laboratories, Burlingame, CA).

CALCIUM DETECTION

Confluent cultures were pre-loaded during 1 h with 5μM Calcium orange-AM (Molecular Probes, Inc.). Thereafter, cells were washed twice with DMEM and treated for 4 h as indicated in each experiment; then, cells were observed under epifluorescence microscopy without mounting solution (only with PBS).

RESULTS

BRIEF EXPOSURE TO STAUROSPORINE COMMITS 3T3-F442A CELLS TO ADIPOSE CONVERSION

One to two day post-confluent cultures of 3T3-F442A cells were incubated with different concentrations of St for 48 h in the presence of either serum-free medium [Salazar-Olivo et al., 1995] or medium containing non-adipogenic serum [Kuri-Harcuch and Green, 1978]. Thereafter, cultures were rinsed with serum-free DMEM and restored to the original medium for a total of 6 days from the beginning of the treatment with St. The number of adipose cell clusters in the cultures after treatment with St or hGH, a known substance that promotes commitment of 3T3-F442A cells into adipocytes [Morikawa et al., 1982, 1984; Nixon and Green, 1984] were counted to quantify commitment into differentiation. Those cells that were committed to adipose differentiation during treatment subsequently formed adipocyte clusters, and since each cluster arises from the selective multiplication and differentiation of one parental cell [Pairault and Green, 1979], the number of adipocyte clusters should correspond to the number of committed cells. Glycerophosphate dehydrogenase (GPDH) activity was also measured as differentiation marker.

St promoted maximum adipose conversion in both serum-free medium and medium containing non-adipogenic serum, as compared to hGH (Fig. 1); we may conclude that St alone, in the absence of other adipogenic factors, was sufficient to induce adipose differentiation of 3T3-F442A cells. Since the effectiveness of the drug seemed to vary according to the lot used, the optimal concentration of St had to be determined for each new batch of the chemical; it was always between 5 and 11 nM, as indicated.

In order to determine the time-course of commitment to adipose differentiation, confluent cells were cultured with non-adipogenic medium for 144 h. St or hGH were added at zero time for the indicated times, after which the medium was replaced with non-adipogenic medium without the drugs; adipose cell clusters were counted at the end of the experiment. It was found that 4 h of exposure to St were sufficient to promote maximum commitment to adipogenesis (Fig. 2A); about 60% of maximum commitment was attained within only 2 h of exposure. Cells cultured as above but treated with 25 ng/ml of hGH as an adipogenic hormone showed that cells took about 24–36 h for commitment (Fig. 2A), which is in agreement with our previously reported results [Castro-Muñozledo et al., 2003].

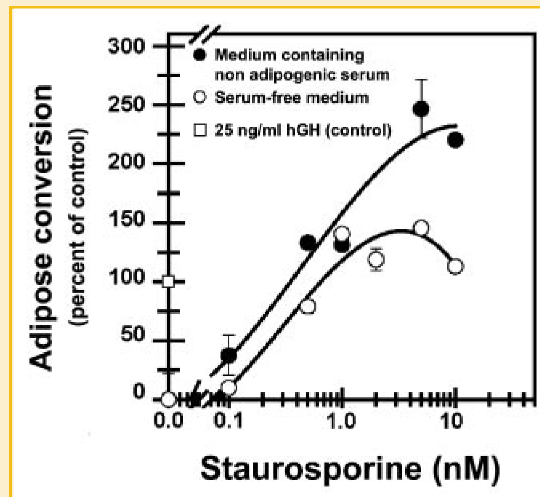


Fig. 1. Adipose conversion of 3T3-F442A cells induced by staurosporine. One day post-confluent 3T3-F442A cells were cultured with serum-free (○) or non-adipogenic medium (●) containing various concentrations of staurosporine for 48 h. Parallel cultures in non-adipogenic medium were also induced to differentiate with (□) 25 ng/ml of hGH for 48 h, as control. Thereafter, all cultures were continued in serum-free or non-adipogenic medium for 6 more days until the end of experiments to determine adipose conversion by staining of intracellular lipids.

To determine the time-course of phenotype expression, parallel cultures were treated as above and the activity of the lipogenic enzyme GPDH, as early marker of phenotypic expression, was quantified in cell extracts at the indicated times. By extrapolating the linearity of the curve to the x-axis, it was calculated that the activity of GPDH began to increase in a time-dependent manner, at about 70 h from the beginning of St incubation; whereas in the case of the cells treated with hGH, the increment began at 90 h after the beginning of the treatment (Fig. 2B). These data, analyzed in a semi-log graphic by regression analysis (not shown), demonstrated that St not only reduced time of commitment, but it also reduced, for about 18–20 h, the time for appearance of the new phenotype, as compared with cultures treated with hGH.

A DESTABILIZER OF COMMITMENT: RETINOIC ACID

Retinoic acid (RA) prevents adipose differentiation of 3T3-F442A cells grown in the presence of medium supplemented with either FBS or GH [Kuri-Harcuch, 1982; Castro-Muñozledo et al., 1987, 2003]. In order to analyze the nature of this effect, confluent cultures were exposed to St in non-adipogenic medium, with or without 10^{-5} M RA for varying periods of up to 8 h. The cells were then washed twice and changed to non-adipogenic medium devoid of retinoic acid, and adipose conversion was then quantified. Addition

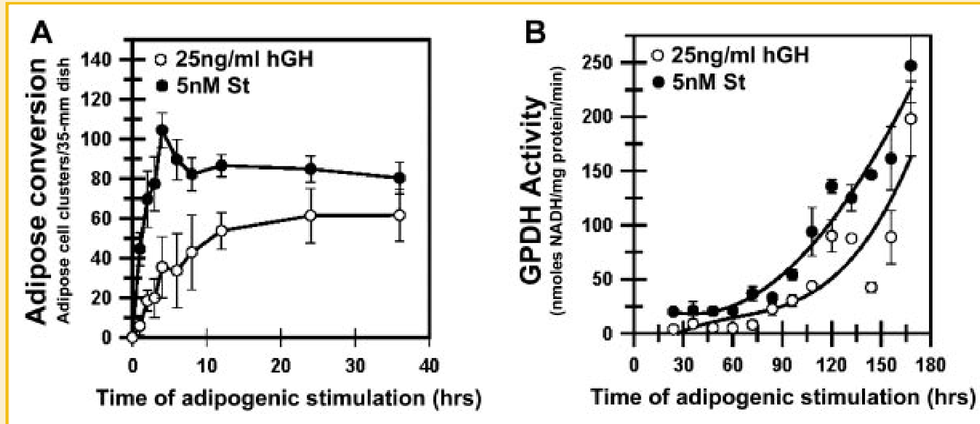


Fig. 2. Time-course of commitment to adipose differentiation induced by St or hGH. A: One day post-confluent cultures were incubated with non-adipogenic medium for 144 h containing 5 nM St (●) or 25 ng/ml hGH (○) for the indicated times, after which cultures were rinsed twice with serum-free medium and further incubated in non-adipogenic medium without St or hGH. At the end of the experiments, cultures were fixed, stained, and the number of adipose clusters/35-mm dish was determined. B: Time-course of GPDH activity during stimulation with St (●) or hGH (○); parallel cultures to those described in (A) were extracted at the indicated times to assay GPDH activity.

of RA during (Fig. 3A) or after (Fig. 3B) St incubation for various periods of time prevented adipose conversion. In order to identify the time at which committed cells were no longer sensitive to the suppressive action of RA, the experiment was repeated by allowing various intervals for non-adipogenic medium after St treatment but before the addition of RA. Figure 3C shows that even if RA addition was delayed for up to 36 h after St, the cells remained unable to undergo adipose conversion. Only when RA addition was delayed for more than 48 h, its ability to inhibit adipose differentiation declined (Fig. 3C). The inhibitory effect of RA was lost when it was removed from the medium, but the cells had to be re-incubated with St or adipogenic conditions in order to become committed to adipogenesis (Table I). These studies showed that commitment of the cells by St consists of two stages: during the first stage, commitment is induced by St, and during the second, commitment is stabilized and no longer required St. The second stage still can be destabilized by the addition of retinoic acid; however, it becomes stable after 48 h of St treatment and can no longer be prevented by retinoic acid.

THE FUNCTION OF GLYCOGEN SYNTHASE KINASE 3β

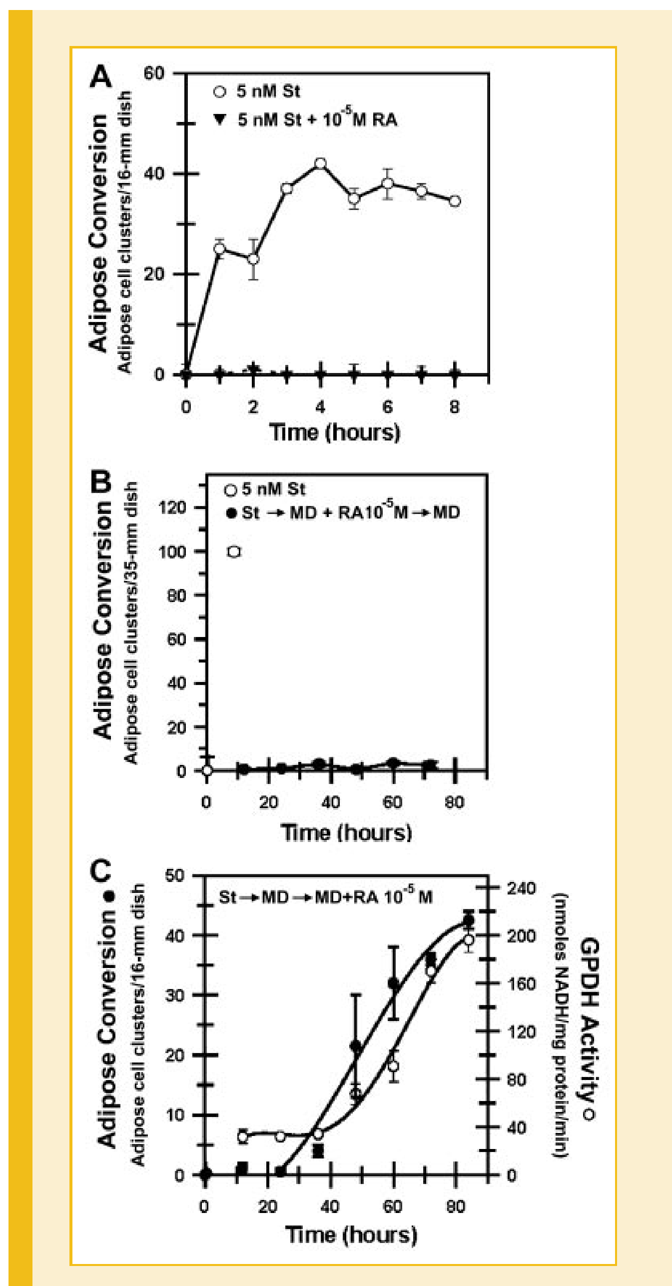
It is well known that glycogen synthase kinase-3β (GSK3β) is activated by staurosporine as was evidenced by reduced phosphorylation on $Ser^{21/9}$ [Koivisto et al., 2003]. It is also well known that this kinase is strongly inhibited by Lithium Chloride [Zhang et al., 2003], which has been widely used to study the role of GSK3β in various cell types. To determine if GSK3β is necessary for commitment, 1–2 day post-confluent cultures maintained in non-adipogenic medium were treated for 4 h with 11 nM St, in the presence or absence of 75 mM LiCl. Then, the cultures were restored to non-adipogenic medium. The extent of adipose differentiation was quantified 6 days later. LiCl suppressed adipose differentiation when added together with St (Table II). Since the amount of LiCl required to inhibiting GSK3β activity varies according to the cell type; we found that 75 mM LiCl was the optimal concentration to inhibit conversion in 3T3-F442A cells; lower concentrations exerted only partial inhibition (results not shown). The inhibitory effect of LiCl on St-dependent commitment is reversible; cells that were exposed simultaneously to St and LiCl for 4 h became committed and the adipose conversion was fully restored when LiCl was removed and incubation continued in the presence of St alone (Table II). Since adipose conversion was fully restored, the inhibition of commitment by LiCl was not due to induction of apoptosis or to cytotoxicity of LiCl. Moreover, inhibition of St-dependent commitment was not due to changes in medium osmolarity since incubation of cultures in the presence of 75 mM NaCl did not block adipose differentiation.

The fact that the role of GSK3β is more complex than anticipated was revealed by the following experiment. Cultures were induced to commitment by incubation with St for 4 h. At the end of the incubation, medium containing St was replaced with medium containing LiCl. This exposure of cells to LiCl after they were induced to commitment by St resulted in complete inhibition of adipose differentiation (Table II). Since LiCl was present only from 12 to 48 h after induction to commitment, a time that corresponds to the second stage of stabilization of commitment and clonal expansion of committed cells, we may conclude that GSK3β activity is required not only for induction of commitment, but also for its subsequent stabilization and clonal amplification. LiCl was not added to cultures after 48 h of induction by St, and therefore it was not explored if GSK3β is also required during phenotype expression.

CALCIUM MOBILIZATION IS ALSO REQUIRED FOR COMMITMENT

GSK3β activation depends on transient increases in intracellular calcium concentrations [Hartigan and Johnson, 1999; reviewed by Grimes and Jope, 2001]. In order to evaluate changes in the intracellular calcium concentration, confluent cultures of 3T3-F442A cells were preloaded for 1 h with 5 μM calcium orange-AM, a calcium fluorescent indicator, and then they were exposed for 4 h to St in non-adipogenic medium. Figure 4A shows that intracellular

calcium concentration increased during St treatment, suggesting that increase in cellular Ca^{2+} might be necessary for commitment to adipose cells. Then, we studied if calcium mobilization was mediated by calcium channels. The 3T3 cells express two types of calcium channels: L-type and T-type voltage-sensitive calcium channels [Chen et al., 1988]. The T-type channels have an important role in various differentiation processes: they are down-regulated during neuronal differentiation [Bertolesi et al., 2003], over-expressed in neuro-endocrine differentiation [Mariot et al., 2002], and involved in the early stages of muscle differentiation [Berthier et al., 2002]. Therefore, the effect of three inhibitors selective for T-type channels was tested. These inhibitors were amiloride, which at concentrations lower than 500 μM inhibits only the T-type channels [Tang et al., 1988], mibefradil with an IC_{50} of 1.5 μM [Martin et al., 2000], and nickel chloride ($NiCl_2$) with an IC_{50} of 250 μM for the α1G subunit of the T-type channels [Lee et al., 1999]. Preadipocytes were cultured as above and incubated for 4 h with St, with or without 400 μM amiloride, 250 μM $NiCl_2$, or 15 μM mibefradil. At the end of the incubation, cultures were rinsed twice with DMEM and changed to non-adipogenic medium for up to 144 h. Table III shows that these blockers partially inhibited adipose conversion, between 20% and 40%, when they were added during induction of commitment with St (Table III). However, when the blockers were added at a time that corresponds to phenotypic expression, 48–72 h after induction with St, neither of them inhibited adipose conversion (results not shown). These results showed that an increase of intracellular calcium is required only during commitment to adipogenesis, in part owed to the T-type calcium channels.



The expression of the T-type αIG gene, which encodes a subunit of the T-type Ca^{2+} channels, was also examined. The preadipose cells fed with non-adipogenic medium were committed by exposure to 11 nM St during 4 h or maintained in 10% FBS during 6 days to obtain fully differentiated adipocytes. At the end of the treatment, αIG gene expression was analyzed by semi-quantitative RT-PCR. Figure 4B shows that the gene was expressed at similar levels in preadipocytes, in committed cells and in mature adipocytes. At the protein level, immunofluorescent staining with polyclonal antibodies against the α1G subunit (see Materials and Methods Section) demonstrated its presence in preadipocytes, in committed cells and in adipocytes (Fig. 4C). Therefore, it was concluded that meanwhile the T-type α1G activity is essential for Ca^{2+} mobilization; there is no detectable change in the expression of the gene during commitment.

Data reported by other authors [Ntambi and Takova, 1996; Shi et al., 2000], and some of our unpublished experiments have shown that 3T3-F442A cells incubated for 48 h with adipogenic medium and ionophore A23187 did not undergo adipose conversion. However, there is no reason to think that GSK3β activation should be hindered by the addition of Ca^{2+} ionophore A23187, which

Fig. 3. Retinoic acid destabilizes commitment into adipose conversion induced by staurosporine. A: One to two days post-confluent cultures were incubated for 144 h with non-adipogenic medium containing 5 nM St (○), or 5 nM St and 10^{-5} M RA (▼) for the indicated times. At the end of incubation with the drugs, cells were washed twice and changed to non-adipogenic medium without the drugs; adipose conversion was quantified at the end of experiment. B: Post-confluent cultures were incubated with non-adipogenic medium for 144 h and treated with staurosporine during the first 8 h only, to achieve maximal commitment (○); some cultures were then changed to 10^{-5} M RA without St for the indicated times and, afterwards, cells were rinsed and changed to non-adipogenic medium (●); adipose conversion was quantified at the end of experiment. C: Adipose cell commitment is prevented by RA when the retinoid is added to cell cultures before 36 h after incubation with staurosporine. Post-confluent cultures were incubated with non-adipogenic medium for 144 h and treated with St during the first 8 h only, to achieve maximal commitment; then they were changed to non-adipogenic medium for various intervals without St, and they were finally incubated with non-adipogenic medium with the retinoid at the indicated times until the end of experiment. Cultures were extracted at the indicated times to assay GPDH activity (○) or they were incubated to the end of experiment (up to a total of 144 h) to be fixed and stained with Oil Red O to determine the number of fat cell clusters (●).

TABLE I. Adipose Conversion of 3T3-F442A Cells Induced by Staurosporine is Reversibly Inhibited by Retinoic Acid

Initial treatment (8 h)	1st incubation after adipogenic induction	2nd incubation after adipogenic induction	Adipose conversion (adipocyte clusters per 16-mm dish) (±SD)
No treatment			4.0 ± 1.0
St	None	None	42.5 ± 1.5
St + RA	None	None	9.0 ± 2.0
St + RA	St; 8 h	None; 128 h	43.5 ± 3.0
St + RA	hGH; 48 h	None; 88 h	38.5 ± 2.0
St + RA	FBS; 48 h	None; 88 h	51.0 ± 5.0

3T3-F442A cells were grown under non-adipogenic conditions. One-day post-confluent cultures were incubated with non-adipogenic medium for 144 h and treated as shown. After incubation, cultures were changed to the indicated culture conditions in non-adipogenic medium. At end of experiment, cultures were fixed and stained to quantify adipose conversion by the number of adipose cell clusters. Concentrations of chemicals were 5 nM staurosporine (St); 10 μM retinoic acid (RA); 10% (v/v) fetal bovine serum (FBS); 25 ng/ml human growth hormone (hGH).

should further increase Ca^{2+}. One day post-confluent cultures were treated with 11 nM St for 4 h, with or without 1 μM A23187 in order to verify this assumption. Table IV shows that, as expected, A23187 added during induction of commitment with St did not inhibit adipose differentiation; however, when A23187 was added 12 h or 72 after St exposure, adipose differentiation was completely blocked (Table IV). This late treatment with the ionophore would include clonal expansion and phenotype expression [Castro-Muñozledo et al., 2003]. The addition of the ionophore after commitment with St blocked subsequent differentiation; however, Ca^{2+} mobilization was required during commitment.

DISCUSSION

The 3T3-F442A cells can differentiate to either adipocytes or osteoblasts [Skillington et al., 2002] depending on their exposure to a specific inducer, which shows that they are not yet committed to differentiate only into adipose cells; BMP signaling and retinoic acid cooperate to repress adipocyte conversion and promote differentiation into fully developed osteoblasts [Skillington et al., 2002]. Adipose conversion of 3T3-F442A cells has been mostly studied after exposure of the cells to adipogenic serum or growth hormone [Kuri-Harcuch and Green, 1978; Morikawa et al., 1982; Foster et al., 1988; Doglio et al., 1989; Gurland et al., 1990; Anderson, 1992, 1993; Campbell et al., 1992; Clarkson et al., 1995; reviewed by MacDougald and Lane, 1995; Liao et al., 1999; Piwien-Pilipuk et al., 2001]. These studies did not deal with the early events that lead to commitment [Smal and DeMeyts, 1987, 1989; Foster et al., 1988; Doglio et al., 1989; Catalioto et al., 1990; Gurland et al., 1990; Johnson et al., 1990; Anderson, 1992, 1993; Campbell et al., 1992], and the mechanisms of commitment into adipocytes remained largely unknown.

It is postulated that cell commitment might depend on the activation and/or inactivation of specific genes during an early period after cell interaction with inducers. Unraveling some of the events and mechanisms involved in the regulation of the newly committed state might require the definition of specific times or stages, still unknown in most differentiating systems. Results reported in this paper show that commitment into adipose differentiation consists of two stages: a first stage of induction and a second stage of stabilization, which still can be destabilized by the addition of retinoic acid or other drugs; then the commitment becomes stable after 48 h of induction.

Based on our experiments that are mainly focused on the early stage, we suggest that the possible mechanisms promoting commitment into adipose differentiation involve mobilization of Ca^{2+} ions by, at least in part, T-type calcium channels, which activates GSK3β, which then would phosphorylate and activate C/EBPβ with the consequent induction of PPARγ and C/EBPα, as late and important modulators for the expression of the adipose phenotype [Christy et al., 1991; Zhu et al., 1995; Tang et al., 1999]. Together, these experiments and previous reports which describe that inhibition of GSK3β activates Wnt pathway [Chen et al., 2000], suggest that Wnt signaling should be inactivated in order to induce commitment of 3T3-F442A cells into adipose differentiation. Wnts have been ectopically expressed demonstrating the need of Wnt pathway inactivation to allow adipogenesis [Ross et al., 2000; Longo

TABLE II. Adipose Conversion of 3T3-F442A Cells Induced by Staurosporine is Inhibited by Lithium Chloride

Initial treatment (4 h)	1st incubation after adipogenic induction	2nd incubation after adipogenic induction	Adipose conversion (adipocyte clusters per 35-mm dish) (± SD)
No treatment			6.5 ± 0.7
St	None	None	67.5 ± 11
75 mM LiCl	None	None	0.5 ± 0.7
St + LiCl	None	None	7.5 ± 0.7
St + LiCl	St; 4 h	None; 136 h	64.0 ± 7.0
St	LiCl; 12 h	None; 128 h	0.0 ± 0.0
St	LiCl; 48 h	None; 92 h	0.0 ± 0.0

3T3-F442A cells were grown under non-adipogenic conditions. One-day post-confluent cultures were incubated with non-adipogenic medium for 144 h and treated as shown. After incubation, cultures were changed to the indicated culture conditions in non-adipogenic medium. At end of experiment, cultures were fixed and stained to quantify adipose conversion by the number of adipose cell clusters. Concentrations of chemicals were 11 nM staurosporine (St); 75 mM LiCl.

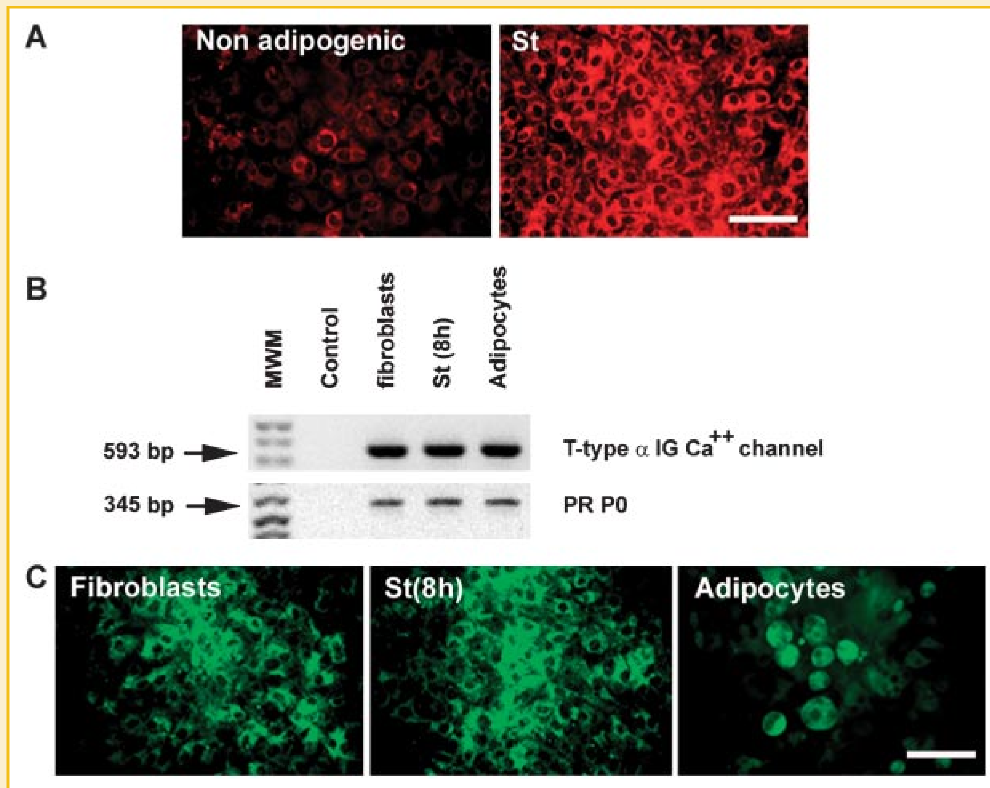


Fig. 4. Calcium mobilization during induction of commitment of 3T3-F442A cells by staurosporine. A: One-day confluent cultures in non-adipogenic medium were pre-loaded for 1 h at room temperature with calcium orange-AM, and then cells were induced with 11 nM St for 4 h in non-adipogenic medium (St). Controls consisted of cells supplemented only with non-adipogenic medium. After treatment, cells were washed twice with PBS and observed by epifluorescence microscopy. Bar = 100 μm. B: RT-PCR analysis of expression of T-type calcium channel subunit αIG gene in total RNA extracts from 3T3-F442A fibroblasts, cells treated with 11 nM St during 8 h or fully differentiated adipocytes. As expected, a specific 593-bp fragment resulted from amplification experiments. Controls consisted in reaction mixture without cDNA, and reaction mixture without primers. As an internal control, primers specific for the ribosomal acidic phosphoprotein P0 (PR P0) were used; the mRNA levels of this protein did not change during adipose conversion. C: Immunostaining of non-differentiated 3T3-F442A cells (Fibroblasts), cells treated with 11 nM St (St) or fully differentiated adipocytes, with antibodies against T-type calcium channel α1G-subunit. Bar = 100 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

et al., 2002]; however, because of the constitutive expression of Wnts, these experiments did not show whether Wnt is inhibited during commitment or at later stages of the differentiation program. We have overcome this limitation during induction of commitment by St, not only by the use of agents to inhibit GSK-3β, an essential component of the Wnt pathway, but also by the use of other agents such as RA, or TNFα which block commitment [Kuri-Harcuch, 1982; Castro-Muñozledo et al., 1987, 2003]; TNFα stimulates Wnt10b expression in a human gastric cancer cell line [Saitoh et al., 2001]. GSK-3β inhibition activates Wnt10b signaling pathway keeping the 3T3-F442A cells in an undifferentiated state through blocking induction of the adipogenic transcription factors C/EBPα and PPARγ [Chen et al., 2000; Ross et al., 2000; Bennett et al., 2002]. It is known that treatment with staurosporine leads to GSK3β activation, as was evidenced by reduced phosphorylation on $Ser^{21/9}$ [Koivisto et al., 2003], and that GSK-3β activity phosphorylates Thr^{179} and Ser^{184} of C/EBPβ, which leads to activation of its DNA-binding function during clonal expansion [Tang et al., 2003, 2005; Zhang et al., 2004]. This promotes the transcription of C/EBPα and PPARγ encoding genes during adipose phenotype expression [Christy et al., 1991; Zhu et al., 1995; Tang et al., 1999]. In addition to its reported role on activation of GSK3β, staurosporine, at low concentrations as the ones used in this study, is a serine-threonine kinase inhibitor which blocks various protein kinase C (PKC) isoforms [Tamaoki et al., 1986]; therefore, experiments are under way in our laboratory

TABLE III. T-Type Calcium Channel Blockers Inhibits Commitment of 3T3-F442A Cells Into Adipose Conversion, Induced by Staurosporine

Treatment (4 h)	Adipose conversion (adipocyte clusters per 35-mm dish ± SD) (%)
No treatment	7.5 ± 0.5 (10)
St	75.0 ± 5.0 (100)
St + $NiCl_2$	44.0 ± 1.0 (58)
St + mibefradil	39.5 ± 2.5 (52)
St + amiloride	60.0 ± 1.0 (80)

3T3-F442A cells were grown under non-adipogenic conditions. One-day post-confluent cultures were incubated with non-adipogenic medium for 144 h. Parallel cultures were treated with staurosporine for 4 h with or without the indicated T-type calcium channels blockers, and then changed to non-adipogenic medium without any blocker until the end of experiment; then, cultures were fixed and stained to quantify adipose conversion by the number of adipose cell clusters. Concentrations of chemicals used were 11 nM staurosporine (St); 250 μM $NiCl_2$; 15 μM Mibefradil; 400 μM Amiloride.

TABLE IV. Adipose Conversion of 3T3-F442A Cells Induced by Staurosporine is Dependent Upon Calcium Entrance

Initial treatment (4 h)	1st incubation after adipogenic induction	2nd incubation after adipogenic induction	Adipose conversion (adipocyte clusters per 35-mm dish) (± SD)
No treatment			1.0 ± 0.0
St	None	None	69.7 ± 13.8
St + A23187	None	None	58.5 ± 8.0
St + A23187	A23187 12 h	A23187; 128 h	1.0 ± 1.0
St	None; 12 h	A23187; 128 h	0.0 ± 0.0
St	None; 72 h	A23187; 72 h	2.5 ± 2.5

3T3-F442A cells were grown under non-adipogenic conditions. One-day post-confluent cultures were incubated with non-adipogenic medium for 144 h and treated as shown. After incubation, cultures were changed to the indicated culture conditions in non-adipogenic medium. At end of experiment, cultures were fixed and stained to quantify adipose conversion by the number of adipose cell clusters. Concentrations of chemicals used were 11 nM staurosporine (St); 1 μM A23187.

to analyze if down-regulation or non-activation of PKCs is also involved in commitment to adipose differentiation.

It was demonstrated that 4 h treatment with St induced commitment into adipocytes and that this commitment could be destabilized by adding retinoic acid during 36–48 h after induction. Since reversibility from retinoic acid inhibition requires a new induction by adipogenic factors or by St, it suggests that the retinoid acid not only inhibits adipose conversion but it might place the cells back into a state susceptible to a new commitment. This would explain the cooperative action of retinoic acid with BMP to bring the 3T3-F442A cells into commitment to the osteoblast lineage [Skillington et al., 2002].

Figure 5 depicts some of the events that are proposed as related to the early inhibition of the Wnt pathway for induction of cell

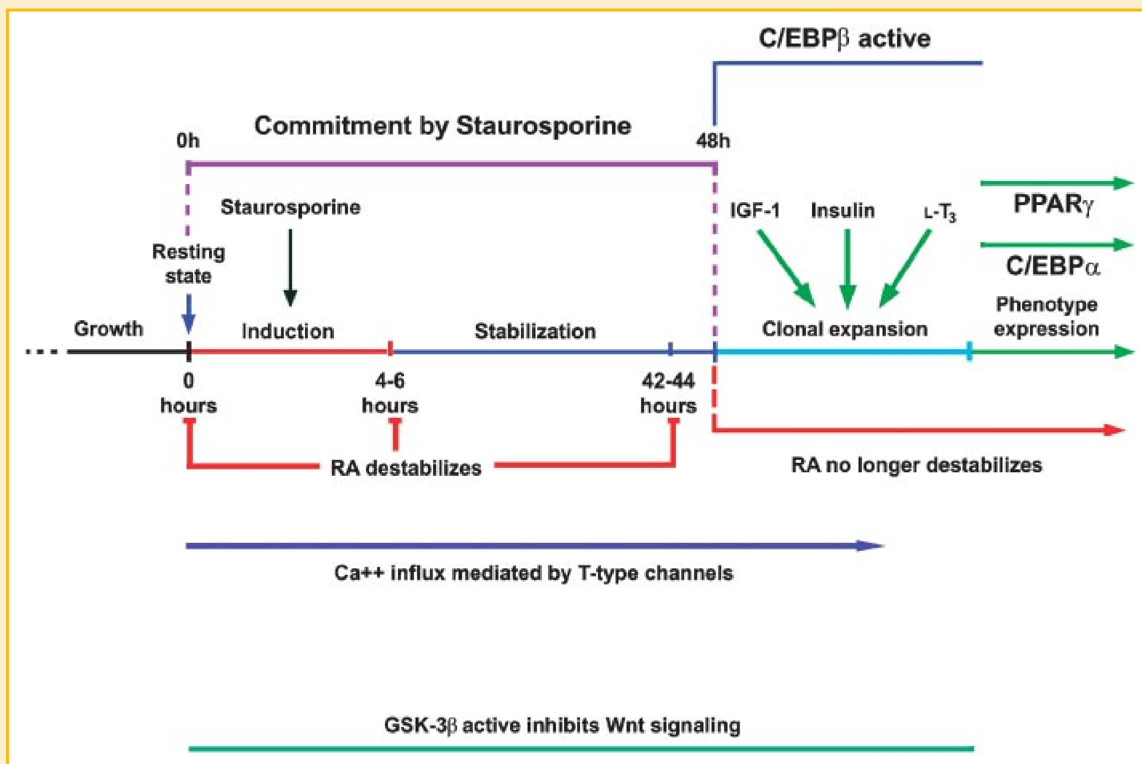


Fig. 5. Diagrammatic representation of the events leading to programming of 3T3-F442A cells into adipocyte differentiation. After growing and reaching resting state, cells should be exposed to adipogenic factors, growth hormone or staurosporine (St) to undergo commitment to differentiation. Treatment of cells with retinoic acid (RA) or TNFα during commitment is reversibly blocked [Kuri-Harcuch, 1982; Castro-Muñozledo et al., 1987, 2003]. We propose that commitment into adipocyte differentiation consists of two different stages: an early stage of induction, in this case mediated by staurosporine during the first 4–6 h, which is blocked by RA; and a late stage of stabilization of commitment which is still sensitive to the inhibitory action of retinoid. During induction, Ca^{2+} influx mediated by T-type channels, activation of GSK-3β and, therefore, inhibition of Wnt signaling is required for commitment. In addition, GSK-3β must remain active during stabilization and clonal expansion of committed cells. After commitment, clonal expansion involves the activation/translocation of C/EBPβ transcription factor, which probably is also present in cells during commitment or part of it. Clonal expansion is positively modulated by IGF-1 [Green et al., 1985; Zezulak and Green, 1985], insulin [Steinberg and Brownstein, 1982; Castro-Muñozledo et al., 2003] and L-T_3 [Flores-Delgado et al., 1987]. After clonal expansion, expression of the adipose phenotype begins, coordinated by transcription factors such as PPARγ and C/EBPα [Tontonoz et al., 1994; Hu et al., 1995; Zhu et al., 1995; Spiegelman, 1998; Tang et al., 1999]; and modulated by insulin and L-T_3, among others. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

commitment. Even though the presence of a second period or stage during commitment, which is still sensitive to the action of certain drugs, was identified, inactivation of the Wnt pathway can not be excluded as essential for stabilization of the committed state. However, since GSK3β is part of the Wnt cascade and since the inhibition of this kinase by LiCl during the second stage of commitment still blocks adipogenesis, we might conclude that Wnt signaling must also remain inactive for stabilization of commitment. This suggests that after induction of commitment, but during the stabilization stage, the generation of new differentiating cells could still be regulated before clonal expansion. The induction and stabilization of commitment before or during clonal expansion is of interest for the study of the mechanisms that might control the early expression of the genes involved in the adipose differentiation program. The identification of these two stages opens the possibility to study early molecular events related to commitment, not only for adipose differentiation but also for other differentiating cell types. Currently, we have experiments underway to examine the early expressed genes that might be related to the induction and stabilization of commitment. Since commitment and selective multiplication of adipocytes are important components of obesity with augmented number of adipocytes and its related metabolic diseases, the identification of some of these early genes might render the possibility of discovering new therapeutic drugs which may prevent an increase in the number of fat cells present in adipose tissue.

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