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EDF-1 downregulates the CaM/Cn/NFAT signaling pathway during adipogenesis

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ABSTRACT

The endothelial differentiation factor-1 (EDF-1) is a calmodulin binding protein that regulates calmodulin-dependent enzymes. In endothelial cells, this factor can form a protein complex with calmodulin. We analyzed the relationship between this factor and the members of calmodulin/calcineurin/nuclear factor of activated T-cells (NFAT) signaling pathway during adipogenesis of 3T3-F442A cells. We found that the expression of *edf1* is upregulated during early adipogenesis, whereas that of calcineurin gene is lowered, suggesting that this pathway should be downregulated to allow for adipogenesis to occur. We also found that EDF-1 associates with calmodulin and calcineurin, most likely inactivating calcineurin. Our results showed that EDF-1 inactivates the calmodulin/calcineurin/NFAT pathway via sequestration of calmodulin, during early adipogenesis, and we propose a mechanism that negatively regulates the activation of calcineurin through a complex formation between EDF-1 and calmodulin. This finding raises the possibility that modulating this pathway might offer some alternatives to regulate adipose biology.

1. Introduction

The 3T3-L1 and 3T3-F442A cells can differentiate into adipocytes when confluent cultures are exposed to adipogenic conditions [1–3]. We have reported that 4 h stimulation with low concentrations of staurosporine (St), an inhibitor of serine and threonine kinases [4], induced rapid adipogenesis of 3T3-F442A cells in non-adipogenic conditions [9]. This adipogenic model allows the study of the early cell response to inducers; St promoted two well-defined stages of adipogenesis before clonal expansion. During the first stage of 4 h, St induced progenitor cells to differentiate, and during the second stage of a subsequent 44 h of stabilization, differentiation continues in the absence of the inducer but can still be reversed by anti-adipogenic substances [5]. This rapid induction allows the study of regulatory mechanisms during very early stages of adipose differentiation [6,7].

There are several evidences that suggest that the Cn/NFATc1 pathway should be involved in regulating adipogenesis. Cn blocks the expression of PPARγ and C/EBPα in 3T3-L1 cells [8], and the activity of NFATc1 is regulated directly by the phosphatase activity of Cn [9]. The sustained activation of NFATc1 inhibited adipose differentiation of 3T3-L1 cells [10]. In C2C12 myoblasts Cn/NFAT signaling upregulates the expression of *rcan1*, which encodes DSCR1/MCIP1 [11] that are family members of endogenous Cn inhibitors, whose expression contributes to downregulation of Cn activity [12].

An important element in the Cn pathway related to Ca^{2+} signaling is the interaction between Cn and CaM, and the intracellular concentration of Ca^{2+} regulates the activity of CaM [13]. Another important element that participates in regulation of CaM activity and CaM-dependent enzymes is the endothelial differentiation factor-1 (EDF-1) [14]. In endothelial cells, EDF-1 is bound with CaM (EDF-1/CaM), through its IQ domain sequestering this complex in the cytoplasm of endothelial cells [15]. In addition, EDF-1 regulates transcription of *pparg* and *cebpa*, as previously described during adipogenesis, and silencing of *edf1* in 3T3-L1 cells blocked adipose conversion, showing its expression is required for the progression of the adipogenic program [16]. However, its role in the CaM/Cn/NFATc pathway has not been studied during the early events of adipogenesis. In this work we explore the role of EDF-1 in the CaM, Cn and NFATc1 cytoplasmic signaling pathway during early adipogenesis.

2. Materials and methods

2.1. Materials

Eagle's medium modified by Dulbecco/Vögt (DMEM), and TRIzol® solution were obtained from Life Technologies-Invitrogen (Carlsbad, CA, USA). Adult bovine serum was from HyClone Thermo Fisher Scientific (Waltham, MA, USA), the adult cat serum was obtained by bleeding domestic animals in accordance with NIH guidelines for animal welfare and protocols approved by the

Abbreviations: Cn, calcineurin; CaM, calmodulin; CsA, cyclosporine A; Dex, dexamethasone; EDF-1, endothelial differentiation factor-1; Iono, ionomycin; NFAT, nuclear factor of activated T-cells; St, staurosporine.

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internal committee for care and management of laboratory animals of CINVESTAV-IPN (CICUAL). EGF was from Lifespan Bioscience. Insulin, d-biotin, human transferrin, triiodothyronine, St, dexamethasone, β-mercaptoethanol, Oil Red O, DMSO and bovine serum albumin were obtained from Sigma–Aldrich Co. (Saint Louis, MO, USA). All other reagents were analytical grade.

2.2. Cell culture and adipose differentiation

The 3T3-F442A cells were cultured at a seeding density of 1.25×10^3 cells/cm^2 [17] in growing medium consisting of DMEM supplemented with 4% adult cat serum, 5 μg/ml insulin and 1 μM D-biotin [3], and maintained at 37 °C with an atmosphere of 10% CO_2 until they reached confluence. For experiments, post-confluent cultures were changed to non-adipogenic medium consisting of DMEM supplemented with 2% adult cat serum, 0.2% adult bovine serum, 5 μg/ml insulin, 1 μM D-biotin, 2 nM triiodothyronine, 40 μM β-mercaptoethanol and 0.01 ng/ml EGF [18]. Adipogenesis was induced with 11 nM St and 250 nM Dex (St/Dex) during 4 h, followed by a medium change to non-adipogenic condition up to 144 h when they reached maximum expression of the adipose phenotype [6]. The 3T3-F442A cultures were fixed with a buffered solution of 4% formalin and stained with Oil Red O, adipose conversion was determined by counting manually the number of adipose clusters under a stereoscope [19]. Those cells that were induced to adipose differentiation during treatment, subsequently formed adipocyte clusters [5].

2.3. RNA extraction and gene expression

Total RNA was extracted using TRIzol® (Invitrogen, Calsbad, CA, USA) from cultured cells. One microgram of total RNA was transcribed into cDNA using SuperSCRIPTII™ (Invitrogen, Calsbad, CA, USA). Relative-quantitative PCR (qRT-PCR) was carried out in the thermocycler CFXTM RealTime System (Bio-Rad, Hercules, CA, USA) using the FastStart Universal SYBR Kit Master (ROX) (Roche, Indianapolis, IN, USA). Reaction conditions and primers used are listed in Table 1. Fold changes were obtained with the software Bio-Rad, CFX Manager 1.6 by the $2^{-\Delta\Delta CT}$ method (Hercules, CA, USA) using the *rplp0* amplification as reference and the beginning of the treatment as control. The amplified products were verified by sequencing using the Big Dye Terminator kit 3.1 (Applied Biosystems, CA, USA).

2.4. Protein extraction and immunobloting

Cells were lysed using the Mammalian Cell Lysis Reagent Proteojet™ (Fermentas Inc. GlenBurnie, MA) supplemented with Complete® protease inhibitor (Roche AppliedScience, Indianapolis, IN). The proteins were separated by 17.5% SDS/PAGE, and immunoblotted with specific antibodies against EDF-1, CaM, NFATc1 and Cn. The proteins were evidenced by luminol™ reagent (Santa CruzBiothecnology, Santa Cruz, CA, USA) and X-ray film (Eastman Kodak Co., Rochester, NY). The densitometric analysis of the immunoblots was done with ImageJ (v1.46) [20].

2.5. Immunoprecipitation of protein complexes

For the antibody-protein G complex, we incubated 50 μl of Protein G Agarose slurry (Invitrogene, Carlsbad, CA) with 3 μg/μl of mouse anti-CaM and 27.75 μg/μl of mouse anti-NFATc1 for 1 h at 4 °C. Subsequently we incubated the mixture with 500 μg/μl of cell lysate at 4 °C with continuous stirring, after 90 min we centrifuged it at 900*g* for 2 min at 4 °C; the supernatant was discarded and the samples washed 3 times with 0.1 ml of lysis buffer. Proteins were dissolved in 60 μl of Laemmli buffer, boiled, centrifuged, and an aliquot of the supernatant was used for immunobloting as described above.

3. Results

3.1. EDF-1, CaM, and Cn gene expression during adipogenesis

We studied the expression of the genes encoding for EDF-1 (*edf1*), CaM (*cam*), and Cn (*ppp3ca*) during the early stages of adipogenesis, after induction of the 3T3-F442A preadipocytes with St/Dex. At the end of experiment, when adipose conversion was complete at 144 h, we determined the number of adipose clusters. Cells cultured without induction did not undergo adipose conversion, as compared with the cultures treated for 4 h with St/Dex, which showed complete differentiation (Fig. 1A). During adipogenesis, *edf1* expression increased by 8 h and continued to do so, reaching a maximum expression by 24 and 48 h (Fig. 1B). The expression of *cam1* and *ppp3ca* decreased early during the first 48 h (Fig. 1B).

EDF-1 protein increased by 8 h (Fig. 2A). Cn decreased at 4 h after induction of adipogenesis, whereas CaM did not change (Fig. 2B and C). Cn content correlated with the expression level of the *ppp3ca* gene. Cn was shown as a 68 and 60 kDa molecular weight bands, which are the known molecular weight for this protein (Fig. 2B). The presence of the lower band could indicate a degradation product by the calpain protease activity on Cn, as previously described in cardiomiocytes [21]. CaM showed the corresponding 17 kDa protein band (Fig. 2C).

3.2. EDF-1 and the Cn/NFATc1 pathway during adipogenesis

Since expression of *ppp3ca*, and Cn content, are down regulated during adipogenesis, we analyzed the expression of *nfatc1* to eval-

Table 1
Specific primers and antibodies.

Gene	ID access	Primers	Antibodies
edf1	NM_021519 PrimerBank 10946942a1	5′-CTGCATCACGACAGGGTGAC-3′ 3′-ATAGTCTGCGATGACTTGCGG-5′	Rabbit anti-Lifespan Bioscience LSC3-1515
cam1	NM_033920 PrimerBank 67532441a1	5′-TGGGAATGGTTACATCAGTGC-3′ 3′-CGCCATCAATATCTGCTTCTCT-5′	Mouse anti-Merck Millipore 05–173
ppp3ca	NM_008913.4 PrimerBank 309129a1	5′-GTGAAAGCCGTTCCATTTCCA-3′ 3′-GAATCGAAGCACCCTCTGTTATT-5′	Mouse anti-Sigma-Aldrich 05–173
nfatc1	NM_001164110.1 Designed for this study	5′-CCACAGGCCTCGTATCAGT-3′ 3′-ATTCTCCAAGTAACCGTGTAGC-5′	Mouse anti-Thermo Scientific MA3-024
rcan1	NM_007475 primerbank 33149234a1	5′-CGCCCAATCCCGACAAACA-3′ 3′-GGTCTCCGTGTCTGGATGATTT-5′	–
prlp0	NM_007475 reported in [8]	5′-AGGCCCTGCACTCTCGCTTTCTGG-3′ 3′-TGGTTCCTTTGGCGGGATTAGTCG-5′	–
Actin	–	–	Monoclonal mouse anti- kindly gift of Dr. Manuel Hernández Hernández

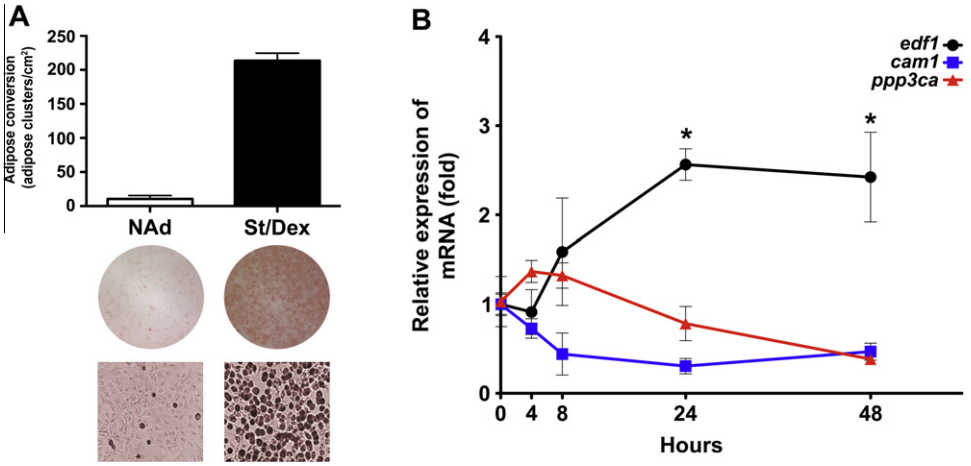

Fig. 1. Expression of *edf1*, *cam1* and *ppp3ca* during adipogenesis of 3T3-F442A cells. (A) Adipose conversion at the end of experiment; non-adipogenic (NAd) and adipogenic (St/Dex) medium. Data are the mean ± s.d. of two independent 35 mm cultures per condition. (B) Expression of *edf1* (●), *cam1* (■), and *ppp3ca* (▲). Statistical significance was identified if the *P* value was lower than 0.05 (∗), data are the mean ± s.d. of two independent experiments by triplicate ($n = 6$).

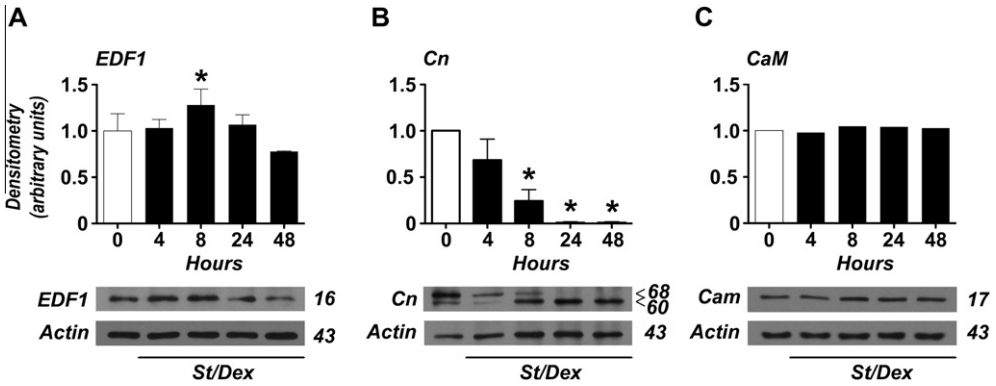

Fig. 2. EDF1, Cn and CaM during adipogenesis of 3T3-F442A cells. (A)–(C) Inmunodetection of EDF-1, Cn and CaM respectively. Cultures were induced with St/Dex and total protein was extracted at different time points. Hundred and fifty microgram of total protein was resolved in 17.5% Tris-Glycine-SDS PAGE, and immunoblotted with specific antibodies. Graph shows densitometric analysis of immunoblotted proteins. Lower panels show a representative assay for each detection, displaying the 16 kDa band corresponding to EDF-1, 68 kDa band for Cn, a 17 kDa band for CaM, and the 43 kDa band for actin as loading control. Statistical significance was identified if the *P* value was lower than 0.05 (*), data are the mean ± s.d. of two independent experiments by duplicate ($n = 4$).

uate whether there is downregulation of the Cn/NFAT signaling pathway, including the expression of *rcan1*, which encodes the protein DSCR1. We found that *nfatc1* expression was decreased in a time-dependent manner by 24 h, and similarly to the expression of *ppp3ca*, whereas *rcan1* expression was not altered and remained constant and similar to pre-adipocytes (Fig. 3A). These results indicated that the signaling pathway of NFATc1 is downregulated during adipogenesis, whereas the pathway that could inhibit phosphatase activity of Cn by DSCR1 remains inactive.

EDF-1 regulates calmodulin-dependent enzymes through binding with CaM (EDF-1/CaM) rendering CaM inactive. Since the expression of the genes encoding Cn and NFAT are downregulated, we evaluated whether EDF-1, whose gene is highly expressed during adipogenesis, could form a protein complex with CaM, contributing to the inactivation of Cn and of the Cn/NFAT pathway. By immunoprecipitation (IP) of CaM and then immunoblot analysis, we found that three proteins seem to be associated in the immunoprecipitated protein complex, EDF-1, CaM and Cn but not NFATc1 (Fig. 3B), whereas IP of NFATc1 did not reveal an association with the other proteins (Fig. 3C). These results show that EDF-1 can associate in a protein complex with CaM, and Cn but not with NFATc1, and these results suggest that EDF-1 is a key element in the CaM/Cn/NFATc1 pathway probably through a sequestration of CaM, and hence inactivation of Cn in the cytoplasm.

3.3. Cn inhibition is necessary for adipogenesis

We attempted to change the activity of Cn through known selective modulators of this protein. We induced the 3T3-F442A preadipocytes to differentiate with St/Dex for 4 h, and we treated them with compounds that are well known modifiers of Cn activity: we exposed cultures for 4–8 h to 2 μM ionomycin (Iono), an ionophore of Ca^{2+} that activates Cn, and therefore should inhibit adipogenesis, 1 μg/ml of cyclosporine A (CsA), a specific inhibitor of Cn, or with the mixture of both drugs (CsA/Iono), as previously described [8]. At the end of experiment we counted the number of adipose clusters and we determined the expression of *edf1*, *nfatc1* and *rcan1* at the indicated times during early adipogenesis.

Cultures treated with Iono showed about 50% inhibition of adipose differentiation as compared to control cultures (Fig. 4A and B). Cultures treated with CsA showed an increased level of adipogenesis, whereas those cultures that were treated with the mixture CsA/Iono had similar differentiation as the induced cultures, suggesting that CsA, reversed the activation of Cn by Iono, and hence cells underwent adipose differentiation (Fig. 4A and B). These results are consistent with our data supporting the need of the inactivation of Cn for the induction of adipogenesis. The expression of the genes, *nfatc1* and *rcan1*, that

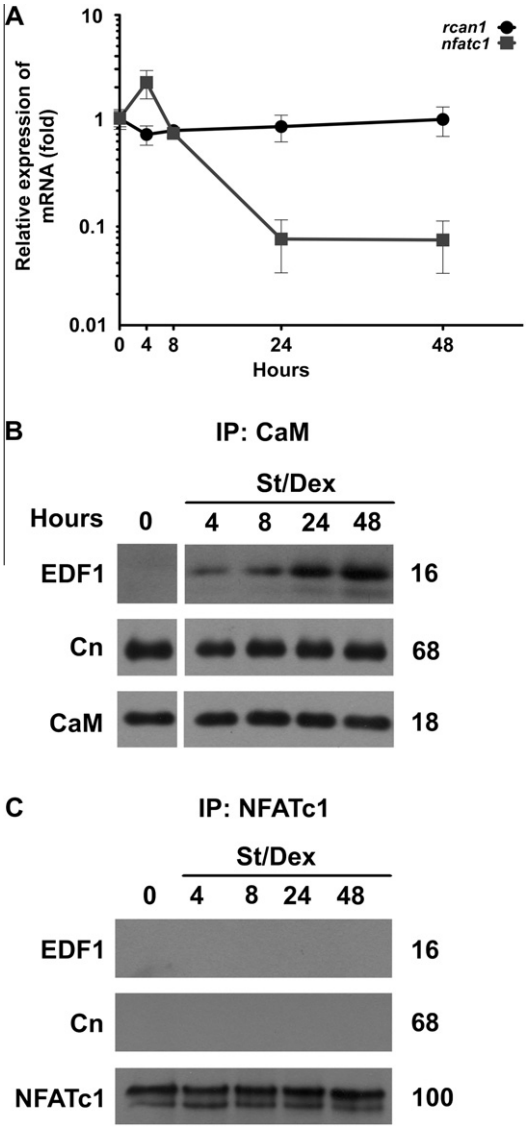

Fig. 3. EDF1 is associated with Cn and CaM during adipogenesis of 3T3-F442A cells. (A) Expression of *nfatc1* (●), *rcan1* (■) during adipogenesis. Gene expression was evaluated by qRT-PCR; data are the mean ± s.d. of two independent experiments by triplicate ($n = 6$). (B) Interaction between CaM with EDF-1 and Cn was determined by co-immunoprecipitation. Total protein was immunoprecipitated with the antibody against CaM as described in section Materials and Methods, and developed in an immunoblot assay with the antibody against EDF-1, anti-Cn and anti-CaM, the latter as a positive control for the assay; same membrane was used for all detections. (C). Interaction between EDF-1, Cn and NFTAc1, cultures as in B were immunoprecipitated with the antibody against NFATc1, the immunoblot detections were performed with antibodies against Cn, EDF-1 or NFATc1. It's clear that there is no interaction between EDF-1 and NFATc1.

are part of the Cn signaling pathway, showed a low level of expression in the cultures induced to differentiate, and high levels of expression in cultures treated with Iono (Fig. 4C and D). Cultures treated with CsA/Iono showed a low level of expression of these two genes, and similar to those of the control cultures (Fig. 4C and D). These results suggest that a downregulation of the Cn/NFATc signaling pathway is necessary for adipogenesis in 3T3-F442A cells. However, the expression of *edf1* increased by incubation with Iono or the combination of CsA/Iono (Fig. 4E) suggesting that the expression of *edf1* should precede modulation of Cn activity, and possibly the existence of a regulatory feedback loop between EDF-1, CaM and Cn for adipogenesis.

4. Discussion

The Ca^{2+} sensor protein CaM is regulated by changes in intracellular Ca^{2+} concentration. An early step necessary during adipose differentiation of 3T3-F442A cells is an increase in the intracellular Ca^{2+} concentration through T-type Ca^{2+} channels [5]. These data raise the possibility that Cn activity is directed by the concentration of Ca^{2+} and CaM, as important steps for adipogenesis. In endothelial cells, EDF-1 is found in both the cytoplasm and the nucleus [14,15,22]. EDF-1 is a highly conserved protein, and in endothelial cells, the cytosolic EDF-1 binds CaM [15,22], whereas in 3T3-L1 cells the role of EDF-1 was explored as co-activator of PPARγ and it is required for transcriptional activation of this adipogenic gene during adipose differentiation [16].

These data, suggested to us that EDF-1 might have a complex role during adipose differentiation. One, at the transcriptional level, as previously described [16], and a second role at early stages of adipose differentiation that could enable adipogenesis, through downregulation of the CaM pathway. It has been suggested that the interaction between CaM and EDF-1 might regulate the intracellular availability of CaM in endothelial cells [15]. Since, NFAT proteins are regulated through the action of the Ca^{2+}/Cn [23–25] it is conceivable that EDF-1 could participate in CaM/Cn/NFAT signaling pathway regulation. An increase in intracellular Ca^{2+} concentration activates Cn, and activation of Cn inhibits adipogenesis [8]. The interaction between CaM and Cn, together with increases in intracellular Ca^{2+} concentration favors the activation of Cn, which blocks the adipose conversion of 3T3-L1 cells [8]. Since an increase in Ca^{2+} concentration is required during early adipogenesis [5], it seems contradictory that an increase in Ca^{2+} concentration would stimulate adipogenesis, and at the same time activate Cn, which blocks adipose differentiation. In addition, signaling through the Ca^{2+}/Cn pathway is required to maintain *nfatc1* transcription, and it is known that inhibition of Cn attenuates its expression [26]. It is plausible that the inactivation of Cn during adipogenesis would take place by downregulating *ppp3ca*, the gene encoding for Cn, or through blocking Cn interaction with CaM, or a combination of both. Since the interaction between CaM and EDF-1 might regulate the intracellular availability of CaM [15], a means to block the interaction of Cn with CaM, blocking Cn activation, would be the sequestration of CaM by EDF-1. Our results showed that, in addition to downregulating the Cn/NFATc expression through down-regulation of *ppp3ca*, a complex formation between EDF-1 and CaM takes place during early adipogenesis, most likely inactivating Cn. By coimmunoprecipitation experiments we found that EDF-1, CaM and Cn, associate during adipogenesis. The combination of a lower expression of *cam*, *ppp3ca* and *nfatc1*, and a protein complex formation dependent on EDF-1 to sequester CaM and inactivate Cn, seem to be a way to ensure a significant inactivation of the Cn/NFATc pathway already present in the cytoplasm of the preadipocytes, and to maintain low levels in the expression of the genes encoding the proteins involved in the CaM/Cn/NFATc signaling pathway.

Our data, at both, the mRNA expression and protein level interaction in the cytoplasm, show that upregulation of *edf1* would impair the Cn/NFATc pathway, allowing the cells to undergo adipogenesis. These changes in the EDF-1/CaM/Cn/NFATc pathway take place after the 4 h induction with St/Dex and mainly during the stabilization stage of adipogenesis, supporting the idea that this signaling does not seem to be involved in the initiation of adipogenesis, rather in the subsequent stages leading to the transit of the cells through stabilization [7]. Since, Cn acts as a blocker of adipogenesis, and since the half-life of this protein is at least 30 h [27], in order to initiate adipogenesis as early as 4 h after induction, sequestering of Cn through the EDF-1/CaM complex becomes

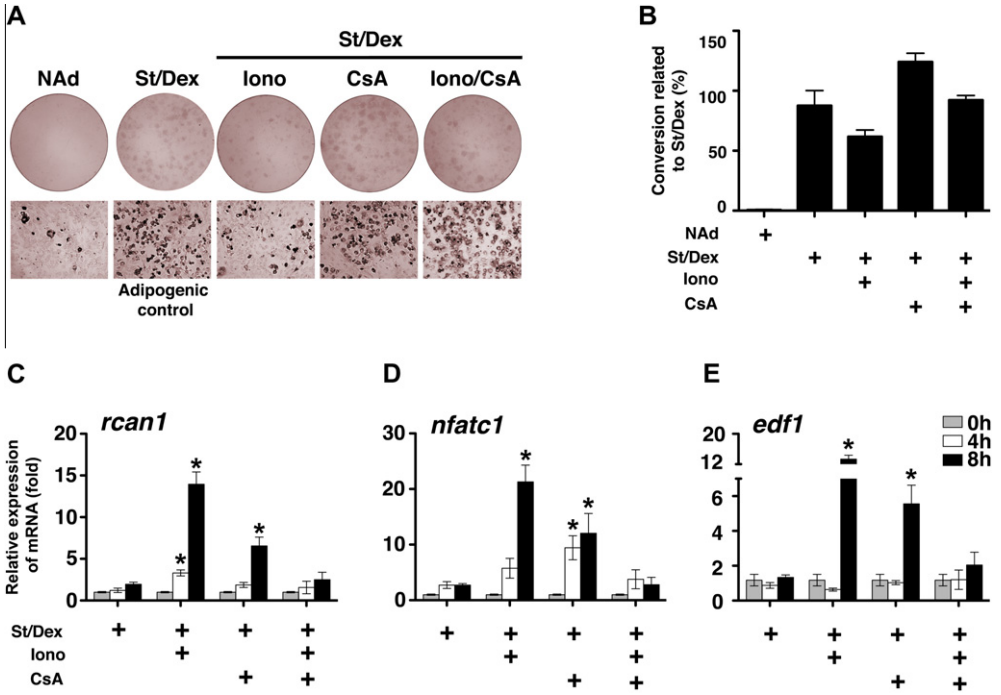

Fig. 4. Cn/NFATc1 during adipogenesis of 3T3-F442A cells. (A) Representative photographs showing adipose conversion of cultures maintained in non-adipogenic (NAd) or adipogenic medium (St/Dex): Parallel cultures were treated 4 h with 2 μM Iono, 1 μg/ml of CsA or with the mixture of both (CsA/Iono). Cultures were fixed at 144 h with formalin and latter stained with Red Oil O. (B) Cultures were treated as in A and the number of adipose clusters was related to the maximal differentiation with St/Dex. Data are the mean ± s.d. of two independent experiments by duplicate ($n = 4$). (C)–(E) Relative expression levels of *rcan1*, *nfatc1*, and *edf1* during the first 8 h during adipogenesis in presence of Iono, or CsA. Cultures were induced with St/Dex or simultaneously exposed to 1 μg/ml of CsA or 2 μM Iono, or with a mixture of both drugs CsA/Iono. The gray bars represent the 0 h, white and black bars represent 4 and 8 h respectively. Statistical significance was identified if the *P* value was lower than 0.05 (*), data are the mean ± s.d. of two independent experiments by triplicate ($n = 6$).

essential. To achieve this goal, the increased level in EDF-1 seems to play an important regulatory role in allowing adipogenesis to proceed. Another element that seems to have an important role initiating adipogenesis related to the NFATc pathway is the function of glycogen synthase kinase-3β (GSK3β) by phosphorylating the Ser-Pro repeats of NFATc that inhibits the ability of NFATc to bind DNA, as it was revealed by inhibition of GSK3β activity with its selective inhibitor LiCl [28]. Our previously described data showed that GSK3β should be early activated to initiate and sustain adipogenesis, and inhibition of this kinase by LiCl blocks adipogenesis in the 3T3-F442A cells [5].

Our data showed that Iono, a Ca^{2+} ionophore, which selectively activates Cn activity, inhibited the early events of adipogenesis. This suggests that the concentration of intracellular Cn, despite the downregulation in *ppp3ca* expression, still plays an important role during adipogenesis. This is also supported with our results showing that CsA, a selective inhibitor of Cn activity, reversed the effect of Iono allowing cells to undergo full adipose differentiation. It is remarkable that in these experiments, the expression of *nfatc1* and *rcan1*, increased significantly as compared to cells undergoing adipogenesis without any treatment with Iono. These results suggested that in a manner not yet well understood, the activity of Cn might be involved in regulating the transcription of some of the early genes related to this signaling pathway, which are involved in blocking adipogenesis.

All these evidences suggest that a critical pathway that has to be blocked to allow for adipogenesis to take place is the CaM/Cn/NFATc pathway. This seems to be accomplished and insured through several actions: First, EDF-1 sequestration of CaM and hence inhibition of Cn pathway. Second, inactivation of NFATc1 ability to bind DNA to promote the transcription of NFAT-dependent genes that could block adipogenesis; this seems to occur through NFATc phosphorylation by GSK3β, which has to be active to initiate and sustain adipogenesis; third, downregulation in the gene expression of Cn and NFATc1. These data strongly suggest that drugs targeted to EDF-1, Cn, or NFATc1 could be helpful tools to regulate adipogenesis, and therefore, have an impact on obesity and some of its complicating diseases. These data also warrants studies to explore the role of EDF-1 not only during adipogenesis, but also on lipid metabolism in mature adipocytes.

Acknowledgments

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