On: 12 July 2015, At: 23:15
Publisher: Taylor & Francis
Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: 5 Howick Place, London, SW1P 1WG

Animal Biotechnology

Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/labt20

Regulation of Stearoyl Coenzyme A Desaturase 1 Gene Promoter in Bovine Mammary Cells

O. di Martino[a], A. Troiano[a], L. Addi[b], A. Guarino[a], S. Calabrò[b], R. Tudisco[b], N. Murru[b], M. I. Cutrignelli[b], F. Infascelli[b] & V. Calabrò[a]

[a] Department of Biology, University of Naples "Federico II", Naples, Italy

[b] Department of Veterinary Medicine and Animal Production, University of Naples "Federico II", Naples, Italy

Published online: 09 Jul 2015.

Animal Biotechnology, 26:251–259, 2015

ISSN: 1049-5398 print/1532-2378 online
DOI: 10.1080/10495398.2015.1022182

Taylor & Francis
Taylor & Francis Group

Regulation of Stearoyl Coenzyme A Desaturase 1 Gene Promoter in Bovine Mammary Cells

O. di Martino,[1] A. Troiano,[1] L. Addi,[2] A. Guarino,[1] S. Calabrò,[2] R. Tudisco,[2] N. Murru,[2] M. I. Cutrignelli,[2] F. Infascelli,[2] and V. Calabrò[1]

[1]*Department of Biology, University of Naples "Federico II", Naples, Italy*
[2]*Department of Veterinary Medicine and Animal Production, University of Naples "Federico II", Naples, Italy*

Stearoyl-Coenzyme A desaturase 1 (SCD1) belongs to the fatty acid family of desaturases. In lactating ruminants, the SCD1 protein is highly expressed in the mammary gland and is relevant for the fatty acid composition of milk and dairy products. Bovine mammary epithelial cells (BME-UV1), cultured *in vitro*, have been proposed as a model to reproduce the biology of the mammary gland. The present study was designed to investigate the responsiveness of bovine SCD1 promoter to serum, insulin, oleic acid, and NFY transcription factor in BME-UV1 cells. A luciferase-based reporter assay was used to monitor the transcriptional activity of the SCD1 promoter region in BME-UV1 cells treated or not with insulin and/or oleic acid. The level of endogenous SCD1 mRNA was evaluated by Real time PCR. Insulin (20 ng/mL) induced a 2.0 to 2.5-fold increase of SCD1 promoter activity. Additionally, the effect of insulin was inhibited by oleic acid, serum components, and NFY enforced expression. Serum and NFY showed no synergistic or additive effect on SCD1 promoter activity suggesting that they repress SCD1 transcription through the same responsive element.

Keywords Bovine mammary cells; Fatty acids; Insulin; Regulation of gene expression; Stearoyl coenzyme a desaturase

INTRODUCTION

Understanding the basis of lipid homeostasis is fundamental for developing new strategies to combat obesity, diabetes, and other diseases of abnormal lipid metabolism. Stearoyl CoA desaturase 1 **(SCD1**, also called Δ^9-desaturase**)** (EC 1.14.99.5) is a short-lived endoplasmic reticulum-bound enzyme that catalyzes the Δ^9-*cis* desaturation of saturated fatty acyl-CoA substrates **(SFAs)** to monounsaturated fatty acids **(MUFAs)**, primarily palmitoyl-CoA and stearoyl-CoA into palmitoleoleyl-CoA and oleyl-CoA, respectively (1). Oleic acid, the main product of SCD1 reaction, is the predominant fatty acid of human adipose tissue triacylglycerols, associating SCD1 with the development of obesity and metabolic syndrome. Moreover, as the SFA/MUFA ratio affects membrane phospholipid composition and fluidity; it has been implicated in obesity, diabetes, neurological disease, skin disorders, and cancer (2). Stearoyl CoA desaturase 1 gene homologs have been identified in a range of species, many of which express multiple isoforms, with SCD1 being the most abundant isoform in lipogenic tissues (3, 4). The SCD1 gene plays an important role in converting *trans-11* C18:1 vaccenic acid into *cis-9, trans-11* C18:2 CLA (5) and is highly expressed in the mammary gland of lactating ruminant (6). Early after parturition, the SCD1 activity in adipose tissue decreases while increasing in mammary gland (7).

Activity and expression of SCD1 have been reported to be regulated by fatty acids, although the responses appear to vary among the species. For instance, oleic acid was shown to reduce rat and bovine SCD1 promoter activity (8, 9) but had no effect on human SCD1 mRNA synthesis (10). Promoter elements that are responsible for the PUFA repression localize with the promoter elements for SREBP-mediated regulation of the SCD gene (11). In *Bos Taurus,* the PUFA response region (PUFA-RE, 60 bp) is essential for the control of SCD1 expression by PUFA (12). This region encompasses the binding sites for Sp1 and NFY transcription factors and the Sterol Response Element (SRE), which is the binding site for the SREBP1 protein. Recently, it has been demonstrated that SREBP1 cooperates extensively with NFY in the control of genes involved in lipid metabolism. Moreover, promoters of genes involved in lipid metabolism were preferentially occupied by the combination of SREBP1 and NFY factors, whereas genes involved in carbohydrate metabolism were enriched among targets of SREBP1 alone (13). In the present study, we have explored the ability of

Address correspondence to V. Calabrò, Department of Biology, University of Naples "Federico II", Via Cinzia, Monte S. Angelo, 80126, Naples, Italy. E-mail: vcalabro@unina.it

oleic acid and serum to repress SCD1 promoter activity in control and insulin-stimulated BME-UV1 immortalized cells that can mimic the *in vivo* response of bovine mammary cells. Moreover, as oleic acid was reported to have no effect on human SCD1 mRNA synthesis (10), we performed similar experiments in MCF7 cells, a human breast cancer cell line previously defined as a model for the study of insulin action on mammalian cell metabolism (14).

MATERIALS AND METHODS

Cell Culture

The BME-UV1 cell line was established from primary bovine mammary epithelial cells by stable transfection with a plasmid, carrying the sequence of the simian virus 40 early region mutant tsA58, encoding the thermolabile large T antigen (15). BME-UV1 cells were provided to us by the Laboratory of Cell Culture-Department of Veterinary Science and Technologies for Food safety at the Veterinary Medicine Faculty (University of Milan, Milan, Italy) and cultured according to Cheli et al. (15). Human breast cancer MCF7 cells were purchased from Cell Line Service (CLS, Berlin-Aldershof, Germany). MCF7 cells were routinely grown into 100 cm^2 plates (Corning Life Science, Corning, NY, USA) as a monolayer culture in Dulbecco's modified Eagle's medium (DMEM) (EuroClone, Pero, MI, Italy) supplemented with 10% (v/v) fetal bovine serum (FBS) (EuroClone), in humidified incubator with 5% CO_2 at 37°C.

Construction of SCD1_PGL3 Reporter Plasmid Containing the Bovine SCD1 Promoter

A 600 bp genomic fragment containing the SCD1 proximal promoter region, -590 from the transcription start site (+1) was amplified by PCR. The amplified region is shown in Fig. 1A and contains the SP1, SRE, and NFY binding sites (Fig. 1A and B). Whole genomic DNA was isolated from leucocytes of cow peripheral blood and used as a template for PCR using bovine SCD1-promoter specific primers: *Forward* 5'GCATGGTACCCCAGTGCCCATC and *Reverse 5'*GGTACCGCGCTGCACGGTGC. The primers included 8 extra-bases (indicated by small caps) to generate protected *XhoI* or *KpnI* restriction sites. The PCR conditions are given as follows: 94°C for 30 seconds, 40 cycles of 57°C for 1 minute, and a final extension step at 72°C for 1 minute. The PCR fragment was gel purified and appropriately digested with *XhoI* or *KpnI* restriction enzyme (Roche Diagnostics, Milan, Italy) to produce cohesive ends. After a step of purification, the digested DNA insert was directionally cloned into the promoter-less luciferase reporter vector, pGL3-Basic (Promega, Madison, WI, USA), using *XhoI* and *KpnI* cloning sites. Ligation reaction was set up at a vector to insert ratio of 1:5, using 50 ng of

(A)

```
          GTG-AGGAGCTCGCGGCAGAGGGAACAGCAGATTGCGCCGAGCCAATGGCAACGGCAGGA
Ovine                    SP1            SRE          NF-Y/NF-1

          GTG-AGGAGCTCGCGGCAGAGGGAACAGCAGATTGCGCCGAGCCAATGGCAACGGCAGGA
Porcine

          GCG-AGGAGCTCGCGGCAGAGGGAACAGCAGATTGCGCCGAGCCAATGGCAACGGCAGGA
Human

          ACGGAGAAGCTAGAGGCAGAGGGGAACAGCAGATTGCGCCTAGCCAATGGAAAAGGCAGGA
Murine

           GTG-AGGAGCTCGCGGCAGAGGGAACAGCAGATTGCGCCGAGCCAATGGCAACGGCAGGA
B. taurus

           GTG-AGGAGCTCGCGGCAGAGGGAACAGCAGATTGCGCCGAGCCAATGGCAACGGCAGGA
Bubalus B.
```

(B)

```
AY241932 (NCBI) Bos Taurus SCD1 promoter region

1261 cgtggtttcc ccactctttc ccaggaaact tccccagtgc ccatccattt gcgaattgcc
1321 cggggccagt cctgggctgg cacgcatccc cgcgccactc cccgactggg tcctctccct
1381 ctcccccagc gcctcagagc ggcagggtgc ccggtagagg cccagcggcc gatgtaagag
1441 aagccgagga gaaagggagg ggagggggtag tgaggagctc GCGGCAgagg gaaCAGCAGA
                                                  SP1          SRE
1501 TTGCGccgag CCAATGGCAa cggcaggacg aggtggcacc aaattccctt cggccaatga
               NFY/NF1
1561 cgcgccagag tctacagaag cccattagca tttccccagg ggcaggggca gaggcagggg
1621 ctgcggcggc caagccgcgg tgtgtgtgca gcatccagtt cttgcttctt cggcccccag
1681 cacgcctcgg cgctctgtct cctcccctct cccgcccatg cggatctccc acggtgaacc
1741 aactctgcgc actttgcccc ttgttggcaa cgaataaaag aggtctgagg aaatacggga
1801 cacagtcacc ccctgccagc gctagccttt aaatccccag cacagcaggt cgggtccgga
1861 caccggtcca gcgcgcaccg Tgcagcggaa ggtcccgagc gcagcgctgc ggatccccac
                        (+1)transcription start site
1921 gcaaaagcag gctcaggaac tagtctacac tcagtttgga ctgccccgaa ctccgctccg
1981 cagtctcagc cccgagaaag tgatcccagt gtctgagagc ccagatgccg gcccacttgc
2041 tgcaagagga ggtgagagct tccgagtaat ggcccccaga ccccgggttc gcgggcgctg
2101 gtgggggcttc tgggcgactc actggagaag agttgagatc accgcgggag aacatagccg
2161 tcttttgcga gttgtgctgc cttcagtttg ttgggaatgg ggattgtaat ttgcaaactt
2221 agatctccaa ctttcgtttc ttaaacttta aagagaaacc tggtcctgcc ggtagccttc
```

FIG. 1. The SCD1 gene promoter. (A) Sequence alignment of the PUFA-RE sequence in the SCD1 proximal promoter region underlined the sequences corresponding to the Sp1 protein binding site, the Sterol Responsive Element, and the NF-Y/NFI consensus. (B) The SCD1 proximal promoter region. The primers used for PCR and subsequent cloning are indicated in italics. Underlining highlights the sequences corresponding to the Sp1 protein binding site, the Sterol Responsive Elemen,t and the NF-Y/NFI consensus. The transcription start site (+1) is indicated in capitals.

pGL3-Basic vector. T4 DNA ligase and 1X ligase buffer were used according to manufacturer's instructions. Positive clones were first selected for the presence of the *XhoI* and *KpnI* 600 bp restriction fragment, by agarose gel electrophoresis, and then sequenced.

The 2.4 Kb fragment containing the p21WAF promoter was retrieved from the p21WAF-CAT plasmid (16) and ligated into the HindIII site of pGL3-basic Vector (Promega) to obtain the p21WAF-Luciferase reporter construct.

The cDNAs encoding human NFYA, NFYB, and NFYC proteins cloned in pBK-CMV expression vector (Stratagene) were provided by Dr. Maria Morasso (NIH, Bethesda, MD, USA).

Transient Transfections and Luciferase Assay

The MCF-7 or BME-UV1 cells were counted and seeded in 6-well plates at a density of 2.5×10^5 cells/well

in 4 mL of complete medium. For luciferase assay, each experimental point was transfected with 1 μg of PGL plasmid (SCD1_PGL3, p21WAF_PGL3, or PGL3 basic vector). Briefly, plasmid DNA was diluted in 250 μL of DMEM serum free and mixed with 2.5 μL of Lipofectamine 2000 (Life Technologies, CA, USA) in 250 μL DMEM serum free. Transfection master mixtures were incubated at room-temperature for 20 minutes, prior to drop-wise addition to MCF7/BME-UV1 cells. Complexes were added to the cells containing 1 mL of DMEM and 0, 5, or 10% FBS/FCS). After 24-hour transfection, the medium was removed, cells were washed twice with PBS and then lysed using the Lysis Buffer (Promega) according to manufacturer's instructions. *Firefly* luciferase activity in cell lysates was measured using Dual Luciferase Assay kit (Promega), according to the manufacturer's instructions. Results were normalized against protein concentration (Bradford Assay, Biorad).

To evaluate the effect of NFY transcription factor, MCF7 cells were co-transfected with 0.5 γ of SCD1_PGL3 basic construct and 0.5 γ of each NFY subunit (NFY-A, NFY-B, NFY-C). Four hours after transfection, the cells were incubated with 0, 5, or 10% FBS, for 24 hours. The promoter activity was evaluated by Luciferase assay.

To evaluate the effect of insulin on SCD1 promoter activity, insulin was added to MCF7 and BME-UV1 cells, at a concentration of 20 ng/mL (3.4 10^{-9} M), 4 hours after transfection. Physiologic concentrations of insulin range between 10^{-8} and 10^{-11} (14); therefore, treatment with 3.4 10^{-9} M insulin is expected to induce a physiological response in mammalian cells.

Cells were then incubated for 24 hours. To evaluate the promoter response to MUFA, oleic acid (≥99% purity, Calbiochem) was added to a concentration of 30 and 50 μM according to a previously published manuscript (17).

Immunoblot

Immunoblots (IB) was performed as previously described (18). The NF-YA (G2, sc-17753) and NF-YB (FL207, sc13045) antibodies (Santa Cruz Biotechnology Inc., Dallas, TX, USA) were used to specifically detect expression of NFYA or B subunits and used at 1:200 dilution. The anti-GAPDH (6C5) was purchased from Santa Cruz Biotechnology Inc.

mRNA Quantification by qPCR

The SCD1 specific transcript was amplified by quantitative PCR. The primers designed for qPCR reaction are: *Forward* TCCGACCTAAGAGCCGAGAA and *Reverse* AGCACAACAACAGGACACCA (NCBI Reference Sequence: NM_173959.4, from 751 to 823, amplified fragment 72 bp). Total RNA was extracted from BME-UV1 cells maintained in culture with FCS 0% or 10%, with or without insulin (20 ng/mL) (Sigma-Aldrich, St. Louis, MO, USA) using Cells to Ct kit (Ambion, Life Technologies, Austin, TX, USA), according to the manufacturer's instructions. To evaluate the response of SCD1 endogenous gene in BME-UV1 cells to MUFA, oleic acid ≥99% purity (Calbiochem, Millipore, Darmstadt, Germany) was added to a concentration of 30 and 50 μM.

For PCR analysis total RNA was isolated using the RNA Extraction Kit from Qiagen (Hilden, Germany) according to the manufacturer's instructions. RNA (2-5 μg) was treated with DNAse I (Promega, Madison, WI, USA) and used to generate reverse transcribed cDNA using SuperScript III (Life Technologies, Carlsbad, CA, USA) and random examers in 20 μL of total reaction volume. All samples in each experiment were reverse transcribed at the same time and the resulting cDNA diluted 1:5 in nuclease-free water and stored in aliquots at −80°C until used.

Real Time PCR with SYBR green detection was performed with a 7500 RT-PCR Thermo Cycler (Applied Biosystem, Foster City, CA, USA). The thermal cycling conditions were composed of 50°C for 2 minutes followed by an initial denaturation step at 95°C for 10 minutes, 45 cycles at 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. For the qPCR, the relative quantification in gene expression was determined using the $2^{-\Delta\Delta Ct}$ method (19). As previously suggested, Eukaryotic translation initiation factor 3 subunit K (EIF3K) and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as an internal control to normalize all data (20). After normalization, the data were presented as fold change relative to the control sample. Appropriate no-RT and non-template controls were included in each 96-well PCR reaction and dissociation analysis was performed at the end of each run to confirm the specificity of the reaction.

Statistical Analysis

All experiments were performed in triplicate and repeated at least two times. Quantitative data were presented as mean ±standard deviation (SD). Comparison between data was analyzed using t-tests. Significant differences were accepted when P values is less than 0.05.

RESULTS

Effect of Fetal Bovine Serum and Insulin on SCD1 Promoter Activity

The sequence of full length SCD1 promoter in *Bos Taurus* (1880 base pairs) is annotated in the (EMBL BANK) with the accession number AY241932, whereas a partial promoter region of *Bubalus bubalis* SCD1 is included in the ENA database (EMBL gene BANK) with

the accession number FM876222. Comparison of the SCD1 proximal promoter region of several mammalian species, using the TFBIND and the TRANSFAC (ver.3.4) software, reveals high homology. The SCD1 proximal promoter sequences of *Bos Taurus* and *Bubalus bubalis* share 97% of identity. Figure 1a shows the PUFA-responsive SCD1 promoter region encompassing the perfectly conserved binding sites for Sp1, the SREBP-1c, and NF-Y transcription factors, as previously described (8).

To evaluate bovine SCD1 promoter activity we generated the SCD1_PGL3 luciferase reporter construct. The bovine SCD1 promoter region including the PUFA-RE was PCR amplified from genomic cow DNA and appropriate primers (see Materials and Methods). The 600 bp amplified fragment was cloned upstream of the luciferase gene in the PGL3 promoter-less vector (Promega, Madison, WI, USA) to generate the SCD1_PGL3 reporter construct.

Cell culture conditions can have a profound impact on intracellular signaling cascade and may be a fondamental source of variability accounting, at least in part, for the conflicting results in the SCD1 literature. For instance, oleic acid was reported to reduce rat and bovine SCD1 promoter activity (8, 9) while having no effect on human SCD1 mRNA synthesis (10). To determine the extent to which serum affects SCD1 promoter activity, MCF7 cells were grown in serum-free or serum-supplemented medium (5–10% FBS) and transiently transfected with the SCD1_PGL3 construct or the p21WAF_PGL3 plasmid, containing a serum-independent promoter (our previous observations). The promoter-less pGL3 basic vector was used to evaluate the background signal. At 24 hours after transfection, cells were collected; whole cell extracts were prepared and subjected to luciferase assay. As shown in Fig. 2a, in presence of serum, the p21WAF promoter activity was unaffected while the SCD1 promoter activity was significantly reduced ($p < 0.02$). In 10% FBS the SCD1 promoter activity was about 4.3-folds lower than in serum-free medium, with a residual activity ranging between 15 and 25%. The background activity of pGL3 basic construct was negligible in both serum conditions ($4.3 +/- 1.5$ RLU, arbitrary units of luciferase activity).

Next, we monitored the effect of insulin on SCD1 promoter activity. The experiments were performed in serum-free condition or 10% serum as this is the most commonly used condition for the growth of mammalian cell lines. Insulin was added to the medium at the concentration of 20 ng/mL. In serum-free medium, insulin treatment caused a 2.5-fold induction of the SCD1 promoter reporter (Fig. 2b). After FBS-supplementation, the SCD1 promoter basal activity was reduced and insulin treatment was unable to evoke any response (Fig. 2b). The results

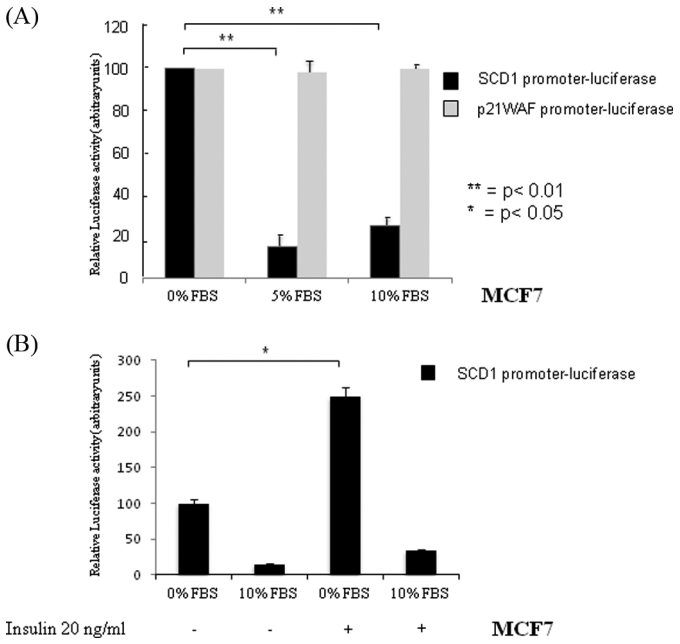

FIG. 2. Effect of FBS on SCD1 promoter activity. (A) The SCD1_PGL3 or p21WAF_PGL3 promoter constructs were transfected into MCF7 cells and treated with different concentrations of serum (0%, 5%, 10%). After 24 hours, the luciferase assay was performed. The p21WAF_PGL3 promoter was used as a serum independent control. The basal activity of the promoters at 0% FBS was fixed as 100%. Each experimental point was performed in triplicate and the results are presented as the mean of three biological replicates. (B) The SCD1_PGL3 promoter construct was transfected into MCF7 cells grown in serum-free or 10% Fetal Bovine Serum. Insulin was added at a concentration of 20 ng/mL. After 24 hours, the luciferase assay was performed. The basal activity of the promoters at 0% FBS was fixed as 100%. Each experimental point was performed in triplicate and the results are presented as the mean of three biological replicates.

demonstrate that serum components exert a strong repression on the SCD1 promoter and that insulin appears to be unable to overcome this repression.

Role of NFY Transcription Factor in the Regulation of SCD1 Promoter Activity

The NFY factor has been suggested to be a PUFA-specific transcription factor for SCD1 gene repression (11). The transcription factor NFY is a hetero-trimeric protein, composed of three subunits NFY-A, NFY-B, and NFY-C. The NF-YB and NF-YC must interact and dimerize for association with NF-YA and consequent binding to CCAAT motifs in the promoter regions of a variety of genes. The NFY interacts with the Sterol Regulatory Element-Binding Proteins (SREBPs). The SREBPs, indeed, are weak transcriptional activators on their own and interact with their target promoters in cooperation with additional regulators, most commonly including one or both NFY and SP1 transcription factors. To investigate

the effect of NFY enforced expression on SCD1 promoter activity, we transiently transfected the SCD1_PGL3 reporter construct into MCF7 cells along with an equal amount of each plasmid encoding NFY subunit A, B, or C. Four hours after transfection, the culture medium was replaced and cells were maintained in serum-free, 5% or 10% FBS-supplemented medium for 24 hours. The expression of transfected NFY subunit A and B was monitored by immunoblot analysis using NFY specific antibodies (Fig. 3A). The SCD1_PGL3 activity was then evaluated by Luciferase assay. As expected, in serum-supplemented media the basal activity of SCD1 promoter was lower than in serum-free medium (Fig. 3A). However, both in absence and 5% serum NFY expression causes a significant decline of luciferase activity ($p < 0.02$) indicating that NFY is a transcriptional repressor of SCD1 gene. However, in 10% serum the residual activity of SCD1 promoter was 50% of the control and NFY caused only a 15% reduction, thereby suggesting that serum and NFY compromise SCD1 promoter activity by acting at the same regulatory element.

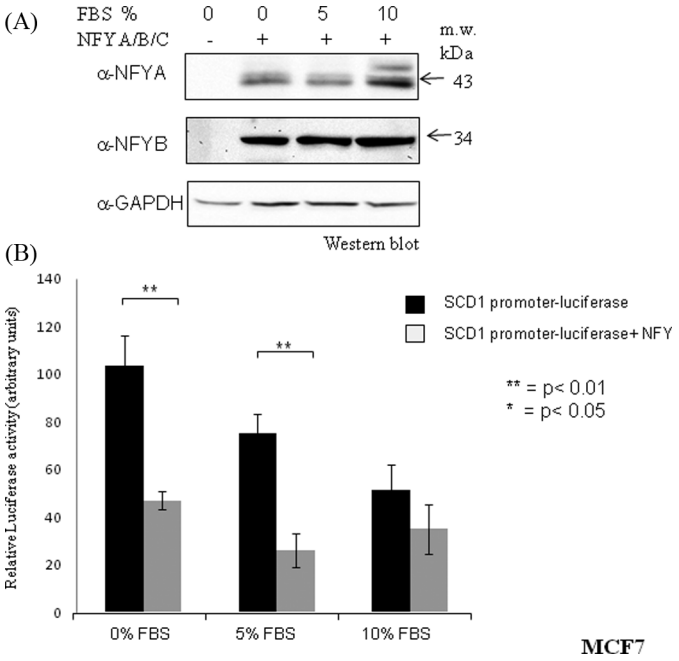

FIG. 3. Effect of NFY transcription factor and on SCD1 promoter activity into MCF7 cells. MCF7 cells were co-transfected with 0.5 μg of SCD-1-PGL3 construct along with 0.5 μg of each plasmid encoding NFY-A, NFY-B, and NFY-C subunits. Cells were grow in serum-free medium or media supplemented with 5 or 10% FBS for 24 hours. (A) Expression of transfected NFYA and NFYB protein was evaluated by immunoblot analysis with specific antibodies. (B) Promoter activity was evaluated by luciferase assay. Each experimental point was performed in triplicate and the results are presented as the mean of three biological replicates.

Regulation of SCD1 Promoter Activity in Bovine Mammalian Cells

The regulation of SCD1 expression in bovine mammary cells affects milk yield and fatty acid profile (21). The SCD1 is induced by insulin (22). It was interesting to study the effect of insulin on SCD1 gene expression in BME-UV1 cells, immortalized, but not transformed, bovine mammary cells that closely mimic the *in vivo* mammary epithelial cells. The promoter reporter construct was transiently transfected into BME-UV1 grown in serum-free, 5% or 10% FCS with or without insulin (20 ng/mL). The BME-UV1 cells were transiently transfected with SCD1_PGL3 construct and after 24 hours whole cell extracts were prepared and subjected to the luciferase assay. According to what was observed in MCF7 cells, serum-addition repressed SCD1 activity in bovine mammary cells (Fig. 4A). Again, we observed a 2.5-fold promoter activation by insulin only in serum-starved cells showing that the repressive activity of serum is dominant over the inductive effect of insulin. To corroborate these data, we decided to examine the level of SCD1 endogenous mRNA in BME-UV1 cells by quantitative Real Time PCR. Cells

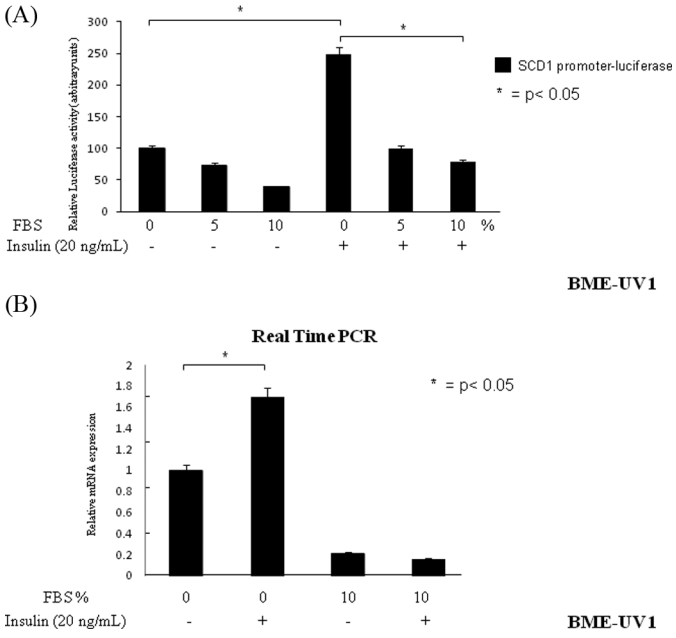

FIG. 4. Effect of serum and Insulin on SCD1 promoter activity. (A) SCD1_PGL3 promoter construct was transfected into BME-UV1 cells treated with the indicated concentrations of serum (0%, 5%, 10%) with or without insulin (20 ng/mL), as indicated. After 24 hours, the promoter activity was evaluated by luciferase assay. Each experimental point was performed in triplicate and the results are presented as the mean of three biological replicates. (B) BME-UV1 cells were treated with the indicated concentrations of serum (0%, 10%) with or without insulin (20 ng/mL), for 24 hours. The SCD1 specific transcript was quantified by Real Time PCR and expressed as the relative amount corresponding to the level expressed in cells grown in serum and insulin-free medium. The results are presented as the mean of three experimental replicates.

were grown in serum-free or 10% FCS medium and treated or not with insulin (20 ng/mL) for 24 hours. Total RNA was isolated and subjected to quantitative PCR using primers to specifically amplify bovine SCD1 mRNA. The level of SCD1 specific transcript was normalized against the EIF3K and GAPDH RNA and expressed as relative amount respect to the sample obtained in serum and insulin-free medium. As shown in Fig. 4B, we confirmed that insulin treatment enhanced SCD1 mRNA expression level only in serum-deprived cells.

Regulation of SCD1 promoter by oleic acid is still controversial. Oleic acids concentrations up to 100 μM were previously demonstrated do not affect MCF7 cell proliferation and viability (23). However, we performed preliminary tests by treating MCF7 and BME-UV1 with increasing amount of oleic acid (15, 30, 50, 80, and 100 μM) for 24 and 48 hours. In each experimental point the cells behave healthy. Moreover, cells lysates were analyzed by immunoblot with antibodies against Caspase3 and PARP and we did not detect signs of apoptosis, neither in terms of Caspase 3 induction nor Poli ADP-ribose polymerase cleavage.

Caspase 3 induction nor Poly (ADP-ribose) polymerase cleavage (data not shown). To check the effect of oleic acid in the control of bovine SCD1 promoter we transiently transfected BME-UV1 cells with the SCD1 promoter-luciferase reporter in serum-free medium supplemented or not with 30 or 50 μM oleic acid.

The combined effect of insulin and oleic acid was checked by adding insulin at a concentration of 20 ng/mL in cells treated or not with oleic acid. After 24 hours of transfection, whole cell extracts were prepared and subjected to the luciferase assay.

As shown in Fig. 5A, oleic acid alone did not significantly change the basal activity of SCD1 promoter, which was instead efficiently activated by insulin. Interestingly, insulin-dependent activation was almost completely abolished by oleic acid addition to the culture medium showing that, similar to serum, the repressive activity of oleic acid overrides the inductive effect of insulin. To substantiate these data, we decided to examine the level of SCD1 endogenous mRNA in BME-UV1 cells by quantitative Real Time PCR. Cells were grown in serum-free medium supplemented or not with 30 or 50 μM oleic acid for 24 hours. The combined effect of insulin and oleic acid was checked by adding insulin at a concentration of 20 ng/mL. Total RNA was isolated and subjected to quantitative PCR using primers to specifically amplify bovine SCD1 mRNA. The level of SCD1 specific transcript was normalized against the EIF3K and GAPDH RNA and expressed as relative amount respect to the sample obtained in oleic acid and insulin-free medium. As shown in Fig. 5B, we confirmed the induction of SCD1 endogenous gene transcription by insulin which was completely suppressed by oleic acid.

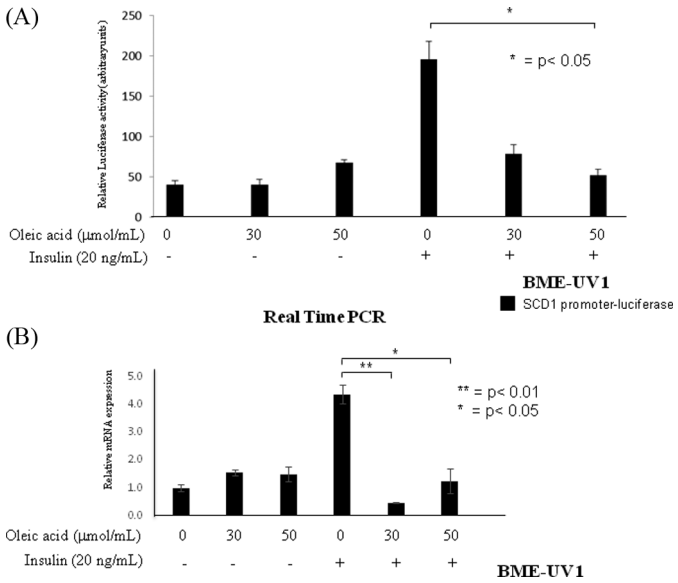

FIG. 5. Effect of insulin and oleic acid on SCD1 promoter activity. (A) SCD1_PGL3 promoter construct was transfected into BME-UV1 cells cultured in serum-free medium with or without insulin (20 ng/mL), as indicated. Oleic acid (30 and 50 μM) was supplemented to the medium as indicated. After 24 hours, the promoter activity was evaluated by luciferase assay. Each the experimental point was performed in triplicate and the results are presented as the mean of three biological replicates. (B) BME-UV1 cells were treated as indicated in (A) for 24 hours. The SCD1 specific transcript was quantified by Real Time PCR and expressed as the relative amount respect to the level expressed in cells grown in serum-free medium without insulin and oleic acid. The results are presented as the mean of three experimental replicates.

DISCUSSION

Alignment of the highly conserved region of bovine SCD1 promoter region shows the expected high homology of the putative PUFA-RE between different farm animal species. This region is known to be involved in the response of SCD promoter to insulin, fatty acids and sterols (24). The PUFA-RE contains binding sites for SREBP1, NFY, and SP1 transcription factors. Genome-wide analysis of promoter co-occupancy in human liver cells have recently shown that SREBP1 cooperates extensively with NFY and SP1 throughout the genome, thereby suggesting that the regulatory circuitry among SREBP, NFY, and SP1 is highly interconnected. Concerning the metabolic pathways, combination of all three factors was reported to be involved in the control of cholesterol biosynthesis and aminoacid activation while the combination of SREBP1 and NFY alone was shown to regulate lipid metabolism and RNA processing (13).

The expression of the SCD l gene is under complex control mechanisms such as hormones and, possibly, intermediates of carbohydrate and fat metabolism; therefore, it is difficult to dissect all the agents that regulate SCD1 gene transcription by *in vivo* models. The aim of this study

was to investigate the responsiveness of bovine SCD1 promoter to insulin, oleic acid, and NFY in BME-UV1 cells, a potential *in vitro* model for studying biotransformation in bovine mammary gland.

In the mammary gland of ruminants, SCD1 is known to be responsible for the production of about 63–97% of c9t11 CLA coming from vaccenic acid as estimated using either direct (13C-labelled fatty acids) or indirect methods (inhibition of SCD by sterculic acid or duodenal and milk FA flows) (25). The SCD1 activity can be measured by comparing the product/substrate ratios of certain fatty acids. There are four main products of SCD1 activity in the mammary gland of ruminants: c9C14:1, c9C16:1, c9C18:1, and CLA, which are produced from C14:0, C16:0, C18:0, and trans11 C18:1, respectively. According to Lock and Garnsworthy (26), the best indicator of SCD1 activity is the c9C14:1/C14:0 ratio because all of the C14:0 in milk fat is produced via de novo synthesis in the mammary gland; consequently, desaturation is the only source of C14:1. Increasing c9C14:1/C14:0 ratio values would indicate an increase of SCD1 activity.

The regulation of SCD1 by dietary factors has been largely investigated in rodents (11), while in ruminants the results are conflicting. Ahnadi et al. (27) and Harvatine and Bauman (28) found a depression of mammary SCD1 mRNA abundance when lactating cows were fed protected PUFA. Researches affected on goats showed that the supplementation of sunflower seed oil (29) and linseed oil (30) did not affect both SCD1 expression and/or activity in maize silage-based diets; whereas, the same supplementation to diets based on grass hay decreased only the SCD1 activity (31). Similar results have been reported supplementing soya beans to lucerne hay-based diets (32). Finally, supplementing grass hay-based diets with formaldehyde-treated linseed decreased mammary SCD1 mRNA without effect on the SCD1 activity (32).

Tudisco et al. (33) reported higher SCD1 expression in the somatic cells of milk yielded from goats bred according to either organic system than those bred in stable. The authors justify the results for a higher amount of both C18:2 and C18:3 ingested by organic group than the stable group, as registered in previous research (34, 35), thus probably resulting in an up-regulation of the SCD expression.

Bernard et al. (36) evaluated the importance of interactions between the composition of the basal diet and lipid supplement with the implication that specific PUFA escaping metabolism in the rumen or specific biohydrogenation intermediates may inhibit SCD1 activity via transcriptional or post- transcriptional regulatory mechanisms.

More recently, Tudisco et al. (37) reported that the grazing season as well as lactation stage can affect the SCD1 mRNA abundance determined from milk somatic cells with values that progressively decreased from April until June, increased in July, and decreased again in August.

In keeping with previous findings (38), we found that insulin treatment induces a significant increase of SCD 1 gene promoter activity in BME-UV1 cells, providing further evidence of its pro-lipogenic role. However, attention must be paid to the evaluation of SCD1 promoter regulation in serum-supplemented cell culture as the repressive effect of serum on SCD1 promoter activity overcomes induction by insulin and this was consistently shown both in human MCF7 and bovine BME-UV1 cells. Remarkably, oleic acid was also able to repress SCD1 promoter activation only in insulin treated cells thereby providing a possible explanation of the controversial literature about the inhibitory effect of oleic on SCD1 expression (11). We confirmed our data on SCD1 endogenous transcription of BME-UV1 cells, thereby demonstrating that our reporter system truly reflects the response of the endogenous SCD1 gene promoter and can be a useful tool to investigate the modulation of SCD1 promoter activity by nutrients and extracellular stimuli. Finally, our study provides evidences that NFY enforced expression represses SCD1 promoter activity. It has to be mentioned that Tabor and coworkers (22) reported that NFY transcription factor is a SCD1 transcriptional activator in adipocytes; our data are in contrast with Tabor's conclusion and this might depend on the specific cell type and growing condition used. However, whether NFY works in cooperation or not with other transcription factors, deserves more attention and will be the subject of further investigation.

ACKNOWLEDGMENT

We thank Prof. Antonella Baldi of the Laboratory of Cell Culture. Dipartimento di Scienze Veterinarie per la Salute, la Produzione Animale e la Sicurezza Alimentare, University of Milan, Italy for providing the BME-UV1 cells.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

FUNDING

This work was supported by "BOVLAC" project. PSR 124, 2007-2013 Regione Campania "Cooperation for development of new products, processes and technologies for agriculture, food and forests".

REFERENCES

1. Ntambi JM. The regulation of stearoyl-CoA desaturase (SCD). Prog Lipid Res 1995; 34:139–150.

2. Paton CM, Ntambi JM. Biochemical and physiological function of stearoyl-CoA desaturase. Am J Physiol Endocrinol Metab 2009; 297:28–37.
3. Flowers MT, Groen AK, Oler AT, Keller MP, Choi YJ, Schueler KL, Richards OC, Lan H, Miyazaki M, Kuipers Kendziorski CM, Ntambi JM, Attie AD. Cholestasis and hypercholesterolemia in SCD1-deficient mice fed a low-fat, highcarbohydrate diet. J Lipid Res 2006; 47:2668–2680.
4. Bionaz M, Loor J. Gene networks driving bovine milk fat synthesis lactation cycle. BMC Genomics 2008; 9(1):366. doi: 10.1186/1471-2164-9-366
5. Bauman, DE, Baumgard LH, Corl BA, Griinari JM. Biosynthesis of conjugated linoleic acid in ruminants. Proc Am Soc Anim Sci 1999. Available at: http://jas.fass.org/cgi/content/abstract/77/E-Suppl/1-ae. Accessed Dec. 5, 2005.
6. Ward RJ, Travers MT, Richards SE, Vernon RG, Salter AM, Buttery PJ, Barber MC. Stearoyl coenzyme A desaturase mRNA is transcribed from a single gene in the ovine genome. Biochim Biophys Acta 1998; 1391:145–156.
7. Griinari JM, Corl BA, Lacy SH, Chouinard PY, Nurmela KV, Bauman DE. Conjugated linoleic acid is synthesized endogenously in lactating dairy cows by Δ9-desaturase. J Nutr 2000; 130:2285–2291.
8. Zulkifli RM, Parr T, Salter AM, Brameld JM. 2010. Regulation of ovine and porcine stearoyl coA desaturase gene promoters by fatty acids and sterols J Anim Sci M 2010; 88:2565–2575.
9. Keating AF, Kennelly JJ, Zhao F. Characterization and regulation of the bovine stearoyl-CoA desaturase gene promoter. Biochem Biophys Res Commun 2006; 200:763–768.
10. Bené H, Lasky D, Ntambi JM. Cloning and characterization of the human steroyl-CoA desaturase gene promoter: Transcriptional activation by sterol regulatory element binding protein and repression by polyunsaturated fatty acids and cholesterol. Biochem Biophys Res Commun 2001; 284: 1194–1198.
11. Ntambi JM. Regulation of stearoyl-CoA desaturase by polyunsaturated fatty acids and cholesterol. J Lipid Res 1999; 40:1549–1558.
12. Waters KM, Miller CW, Ntambi JM. Localization of a polyunsaturated fatty acid response region in stearoyl-CoA desaturase gene 1. Biochim Biophys Acta 1997; 1349(1): 33–42.
13. Reed BD, Charos AE, Szekeley AM, Weissman SM, Snyder M. Genome-wide occupancy of SREBP1 and its partners NFY and Sp1 reveals novel functional roles and combinatorial regulation of distinct class of genes. PLOS Genetics 2008; 4(7):e1000133. DOI: 10.1371/journal.pgen.1000133
14. Osborne CK, Bolan G, Monaco ME, Lippman ME. Hormone responsive human breast cancer in long-term tissue culture. Effect of insulin. Proc Natl Acad Sci USA 1976; 73(12):4536–4540.
15. Cheli F, Zavizion B, Todoulou O, Politis I. The effect of calcium on mammary epithelial cell proliferation and plasminogen activating system. Can J Anim Sci 1999; 277–283.
16. Calabrò V, Mansueto G, Parisi T, Vivo M, Calogero RA, La Mantia G. The human MDM2 oncoprotein increases the transcriptional activity and protein level of the p53 homolog p63. J Biol Chem 2002; 277(4):2674–2681.
17. Harvey KA, Walker CL, Xu Z, Whitley P, Pavlina TM, Hise M, Zaloga GP, Siddiqui RA. Oleic acid inhibits stearic-induced inhibition of cell growth and pro-inflammatory responses in human aortic endothelial cells. J Lipid Res 2010; (12):3470–3480.
18. Rossi M, De Simone M, Pollice A, Santoro R, La Manti G, Guerrini L, Calabrò V. Itch/AIP4 associates with and promotes p63 protein degradation. Cell Cycle 2006; 5(16):1816–1822.
19. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using Real-Time 436 Quantitative PCR and the 2-DDCT method. Methods 2011; (25):402–408.
20. Kadegowda AKG, Bionaz M, Thering B, Piperova LS, Erdman RA, Loor JJ. Identification of internal control genes for quantitative polymerase chain reaction in mammary tissue of lactating cows receiving lipid supplements. J Dairy Sci 2009; 92(5):2007–2019.
21. Pauciullo A, Cosenza G, Steri R, Coletta A, La Battaglia A, Di Berardino D, Macciotta NP, Ramunno L. A single nucleotide polymorphism in the promoter region of river buffalo stearoyl CoA desaturase gene (SCD) is associated with milk yield. J Dairy Res 2012; 79(4):429–35.
22. Tabor DE, Kim JB, Spiegelman BM, Edwards PA. Identification of conserved cis-elements and transcription factors required for sterol-regulated transcription of stearoyl-CoA desaturase 1 and 2. J Biol Chem 1999; 274:20603–20610.
23. Dailey OD Jr, Wang X, Chen F, Huang G. Anticancer activity of branched-chain derivatives of oleic acid. Anticancer Res 2011; 31(10):3165–3169.
24. Biddinger S, Miyazaki M, Boucher J, Ntambi J, Kahn, CR. Leptin suppresses stearoyl-CoA desaturase 1 by mechanisms independent of insulin and sterol regulatory element–binding protein-1c. Diabetes 2006; 55:2032–2041.
25. Bernard L, Leroux C, Chilliard Y. Nutritional regulation of mammary lipogenesis and milk fat in ruminant: contribution to sustainable milk production. Revista Colombiana de Ciencias Pecuarias 2013; 26:292–302.
26. Lock AL, Garnsworthy PC. Seasonal variation in milk conjugated linoleic acid and D9-desaturase activity in dairy cows. Livest Prod Sci 2003; 79:47–59.
27. Ahnadi CE, Beswick N, Delbecchi L, Kennelly JJ, Lacasse P. Addition of fish oil to diets for dairy cows. II. Effects on milk fat and gene expression of mammary lipogenic enzymes J Dairy Res 2002; 69:521–531.
28. Harvatine KJ, Bauman DE. SREBP1 and thyroid hormone responsive spot 14 (S14) are involved in the regulation of bovine mammary lipid synthesis during diet-induced milk fat depression and treatment with CLA. J Nutr 2006; 136: 2468–2474.
29. Bernard L, Rouel J, Leroux C, Ferlay A, Faulconnier Y, Legrand P, Chilliard Y. Mammary lipid metabolism and milk fatty acid secretion in alpine goats fed vegetable lipids. J Dairy Sci 2005; 88:1478–1489.
30. Bernard L, Bonnet M, Leroux C, Shingfield KJ, Chilliard Y. Effect of sunflower-seed oil and linseed oil on tissue lipid metabolism, gene expression, and milk fatty acid secretion

in alpine goats fed maize silage–based diets. J Dairy Sci 2009; 92:6083–6094.
31. Bernard L, Leroux C, Faulconnier Y, Durand D, Shingfield KJ, Chilliard Y. Effect of sunflower-seed oil or linseed oil on milk fatty acid secretion and lipogenic gene expression in goats fed hay-based diets. J Dairy Res 2009; 76:241–248.
32. Bernard L, Leroux C, Bonnet M, Rouel J, Martin P, Chilliard Y. Expression and nutritional regulation of lipogenic genes in mammary gland and adipose of lactating goats. J Dairy Res 2005; 72:250–255
33. Tudisco R, Calabrò S, Cutrignelli MI, Moniello G, Grossi M, Gonzalez OJ, Piccolo V, Infascelli F. Influence of organic systems on Stearoyl-CoA-Desaturase in goat milk. Small Rumin Res 2012; 106:37–42.
34. D'Urso S, Cutrignelli MI, Calabrò S, Bovera F, Tudisco R, Piccolo V, Infascelli F. Influence of pasture on fatty acid profile of goat milk. J Anim Physiol An Nutrit 2008; 92:405–410.
35. Tudisco R, Cutrignelli MI, Calabrò S, Piccolo G, Bovera F, Guglielmelli A, Infascelli F. Influence of organic systems on milk fatty acid profile and CLA in goats. Small Rumin Res 2010; 88:151–155.
36. Bernard L, Leroux C, Chilliard Y. Expression and nutritional regulation of stearoyl-CoA desaturase fenes in the ruminant mammary gland: relationship with milk fatty acid composition. In: Ntambi JM, ed. *Stearoyl-CoA Desaturase Genes in Lipid Metabolism*. New York: Springer Science+Business Media; 2013:161–193.
37. Tudisco R, Grossi M, Calabrò S, Cutrignelli MI, Musco N, Addi L, Infascelli F. Influence of pasture on goat milk fatty acids and stearoyl-CoA desaturase expression in milk somatic cells. Small Rumin Res 2014; 122:38–43. DOI: 10.1016/j.smallrumres.2014.07.016.
38. Mauvoisin D, Rocque G, Arfa O, Radenne A, Boissier P, Mounier gene C. Role of the PI3-kinase/mTor pathway in the regulation of the stearoyl CoA desaturase (SCD1) expression by insulin in liver. J Cell Comm Signal 2007; 1(2):113–125.