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Mechanism Underlying Long-Term Regulation of 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase During L6 Myoblast Differentiation

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ABSTRACT

3-Hydroxy 3-methylglutaryl Coenzyme A reductase (HMG-CoAR) and its end-products are crucial for insulin-induced differentiation of fetal rat myoblasts (L6) both at early and terminal stages of development. Inhibition of HMG-CoAR activity and reduction of the enzyme levels impair the expression of L6 differentiation markers and prevent myoblast fusion into multinucleated syncytia. The mechanism underlying the modulation of this crucial enzyme so that muscular differentiation can occur is poorly understood. Thus, the aim of this work was to explore the long-term regulation of HMG-CoAR in an attempt to provide a new molecular basis for the control of muscle development. All experiments were performed in L6 rat myoblasts induced to differentiate utilizing insulin. The results indicate the following: (i) at early stages of L6 differentiation, the increase in HMG-CoAR protein levels is probably due to transcriptional induction and a decrease in the enzyme degradation rate; (ii) the subsequent reduction of HMG-CoAR protein levels is related both to an increased degradation rate and reduced gene transcription, as indicated by the rise of Insig-1 levels and the subsequent decrease in the amount of n-SREBP-1; (iii) in the terminal stages of myogenesis, reduced protein levels of HMG-CoAR could be ascribed to the decrease in gene transcription while its degradation rate is not affected. By highlighting the mechanisms involved in HMG-CoAR long-term regulation during myogenesis, this work provides useful information for searching for tools to improve the regenerative ability of muscle tissue and for the development of new pharmacological treatments of myopathies. J. Cell. Biochem. 110: 392–398, 2010.

KEY WORDS: HMG-CoAR; INSIG; MUSCULAR DIFFERENTIATION; SREBP

Products derived from the cholesterol biosynthetic pathway, such as ubiquinone, dolichol, and prenyls, are essential compounds for survival, proliferation, and differentiation of cells [Ogura et al., 2007; Viccica et al., 2007]. Thus, the key rate-limiting enzyme of this pathway [Goldstein and Brown, 1990], 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoAR), must play an important role in these physiological processes.

HMG-CoAR, which catalyzes the conversion of HMG-CoA to mevalonate, a four-electron oxidoreduction [Friesen and Rodwell, 2004], is a highly regulated enzyme, subject to transcriptional, translational, and post-translational control [Xu and Simoni, 2003]. It can induce up to 200-fold changes in enzyme levels as a function of intracellular sterol and cholesterol uptake by Low Density Lipoprotein receptor (LDLr) [Goldstein et al., 2006].

To monitor levels of membrane sterols, cells employ another membrane-embedded protein of the endoplasmic reticulum, (ER)-Scap (SREBP cleavage activating protein), in addition to HMG-CoAR, both containing a polytopic intramembrane sequence called sterol-sensing domain (SSD).

Scap is an escort protein for Sterol Regulatory Element Binding Proteins (SREBPs), membrane bound transcription factors able to induce expression of genes required for the synthesis and uptake of cholesterol, such as *HMG-CoAR* and *LDLr* [Brown and Goldstein, 1997; Horton et al., 2002]. In sterol-deprived cells, Scap binds SREBPs and escorts them from the ER to the Golgi apparatus where SREBPs are proteolytically processed to yield active fragments that enter the nucleus and induce expression of their target genes [Brown and Goldstein, 1999]. When cholesterol builds up in ER membranes, the Scap/SREBP complex fails to exit the ER, the proteolytic processing of SREBPs is abolished and the transcription of target genes declines.

ER retention of Scap/SREBP is mediated by sterol-dependent binding of Scap/SREBP to Insig (INSulin Induced Gene), an ER resident protein [Yang et al., 2002]. Intracellular accumulation of

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sterols induces HMG-CoAR to bind Insig, promoting ubiquitination and proteasomal degradation [Sever et al., 2003].

It has been recently shown that HMG-CoAR and its end-products are crucial for myogenesis both at early and terminal stages in insulin-induced fetal rat myoblast (L6) differentiation [Martini et al., 2009]. Myogenesis is a dynamic process where undifferentiated mononuclear myoblasts proliferate at first, then withdraw from the cell cycle and finally differentiate and fuse to form mature multinucleated muscle fibers. This process is controlled by members of a family of muscle-specific basic helix–loop–helix (bHLH) proteins that, in concert with members of the ubiquitous E2A and myocyte enhancer factor-2 (MEF2) families, activate the differentiation program by inducing the transcription of muscle-specific regulatory and structural genes [Lluís et al., 2006].

The important role of the HMG-CoAR pathway in muscle physiology is demonstrated by the observations that the inhibition of HMG-CoAR activity and transcription and the induction of HMG-CoAR degradation result not only a decrease in Myogenin (Myo) and Myosin Heavy Chain (MHC) protein levels (known as early and late markers of myoblast differentiation), but also inhibit myoblast fusion into multinucleated syncitia [Martini et al., 2009]. Moreover, experimental and clinical studies show that HMG-CoAR inhibitors known as statins, which are widely used in hypercholesterolemia therapies, could cause myopathy characterized by weakness, pain and elevated serum creatine phosphokinase [Christopher-Stine, 2006]. Thus, pathologies characterized by muscular weakness or damage could be improved by stimulating myogenesis through the modulation of HMG-CoAR activity and protein levels. Although the crucial role of HMG-CoAR in differentiating L6 cells has been recently demonstrated, the molecular mechanisms of long-term HMG-CoAR regulation during insulin-induced rat myoblast differentiation is completely unknown. In fact, identification of the factors involved in long-term HMG-CoAR regulation could provide useful information in the development of new pharmacological treatments for myopathies. Thus, the aim of this work was to study the steps of such regulation during skeletal muscle differentiation in an attempt to find new tools to enhance the regenerative ability of muscle tissue. To analyze the protein network regulating HMG-CoAR, we used the well-characterized insulin-induced L6 rat myoblast differentiation model. In this model, HMG-CoAR long-term regulation and the factors involved in, studied.

MATERIALS AND METHODS

MATERIALS

All materials were obtained from commercial sources and were of the highest quality available. All materials with no specified source were obtained from Sigma–Aldrich (Milan, Italy).

CELL CULTURE

Rat L6 skeletal muscle cells were used in all experiments. Undifferentiated L6 myoblasts were purchased from ATCC (Manassas, VA) and were cultured in DMEM containing 10% fetal calf serum, L-glutamine (2 mM), gentamicin (0.1 mg/ml) and penicillin (100 U/ml) at 5% CO_2. Cells were plated in six-well plates or 25-cm^2 flasks at a density of 5,000 cells/cm^2 in DMEM containing 10% FBS, grown to ~70% confluence and then stimulated with 10^{-8} M insulin to induce differentiation. To analyze whether long term HMG-CoAR regulation was affected by the modulation of transcription and/or the modulation of protein translation, L6 myoblasts were stimulated with insulin in the presence of actinomycin (ACT) (1 μg/ml) and cycloheximide (CHX) (10 μg/ml), which are transcription and protein translation inhibitors, respectively.

PROTEIN LEVELS ANALYSIS

Protein levels were analyzed by Western blotting. Analysis of HMG-CoAR, Insig-1,and nSREBP-1 was performed on cell lysates according to Martini et al. [2007]. Twenty micrograms of protein were resolved by 12% (for Insig-1) and 10% (for HMG-CoAR and nSREBP-1) SDS–PAGE at 100 V for 60 min. The proteins were subsequently transferred onto nitrocellulose membranes for 80 min at 100 V. Membranes were treated with 3% BSA in 138 mM NaCl, 27 mM KCl, 25 mM Tris–HCl, 0.05% Tween-20 (pH 6.8), and probed at 4°C overnight with primary antibodies. Membranes were then incubated with secondary probes for 1 h. Membranes were stripped by Restore Western Blot Stripping Buffer (Pierce Chemical, Rockford, IL) for 10 min at room temperature and then probed with anti-tubulin antibody (MP Biomedicals, Solon, OH). Bound antibodies were visualized using enhanced chemiluminescence detection (GE Healthcare, Milan, Italy). All images were analyzed as arbitrary units by ImageJ (NIH, Bethesda, MD) software for Windows.

Antibodies were obtained as follows: SREBP-1 (Santa Cruz Biotechnology, Santa Cruz, CA), secondary goat anti-rabbit conjugated to HRP (UCS Diagnostic, Rome, Italy); Insig-1 (Novus Biologicals, Littleton, CO), secondary goat anti-rabbit conjugated to HRP (UCS Diagnostic); HMG-CoAR (Upstate, Lake Placid, NY), secondary goat anti-rabbit conjugated to HRP (UCS Diagnostic); Tubulin (MP Biomedicals), secondary goat anti-mouse conjugated to HRP (UCS Diagnostic).

RNA ISOLATION AND QUANTITATIVE RT-PCR ANALYSIS (qRT-PCR)

The sequences for gene-specific forward and reverse primers were designed using the Primer Express program by Applied Biosystems. The following primers were used: for rat HMG-CoAR (GenBank Accession NM_013134), 5′-CCTTGACGCTCTGGTGGAAT-3′ (forward) and 5′-CCTGACATGGTGCCAACTCC-3′ (reverse), for rat Insig-1 (GenBank Accession NM_022392), 5′-TGCAGATCCAGCGGAATGT-3′ (forward) and 5′-CCAGGCGGAGGAGAAGATG-3′ (reverse), and for tubulin (GenBank Accession AB 011679) 5′-GTGGAATGG-ATCCCCAACAA-3′ (forward) and 5′-CCGTGCTGTTTCCGATGAA-3′ (reverse). Total RNA was extracted from L6 cells using TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. To determine Insig-1 gene expression levels, cDNA synthesis and qPCR were performed using a one-step qRT-PCR kit ("SuperScript™ III Platinum®–SYBR® Green One-Step" kit; Invitrogen) according to the manufacturer's instructions. cDNA synthesis and qPCR were carried out as follows: first strand cDNA synthesis was performed at 50°C for 5 min, followed by an automatic hot-start *Taq* DNA Polymerase activation step at 95°C for 5 min, and then by 25 cycles of denaturation at 95°C for 15 s, annealing at 60°C

for 30 s, and elongation at 40°C for 1 min. Gene expression was verified by 2% agarose gel electrophoresis. Each sample was tested in duplicate and the experiment repeated three times.

HMG-CoA REDUCTASE DEGRADATION IN VITRO ASSAYS

L6 myoblasts treated with insulin for 4, 16, 24, and 48 h were suspended in ice-cold 10 mM Tris–HCl (pH 7.4), 150 mM sucrose, sonicated three times and then incubated at 37°C [Pallottini et al., 2004].

The protein concentration was determined as per Lowry et al. [1951]. At certain established times (0, 4, 16, 24, and 48 h), the incubation was blocked by the addition of an equal volume of sample buffer (final concentration 0.125 M Tris–HCl (pH 6.8) containing 10% SDS, 1 mM phenylmethylsulphonyl fluoride); samples were boiled for 2 min, and the proteins were separated by SDS–PAGE on 10% polyacrylamide gels. The proteins were transferred to nitrocellulose membranes, and HMG-CoA reductase was detected using anti-HMG-CoAR antibody (Upstate). Detection of bound antibody was performed using anti-rabbit IgG and the ECL Western blotting Kit (GE Healthcare).

RESULTS

The objective of this work was to understand the mechanisms involved in long term HMG-CoAR regulation during the myogenic process. In other studies, authors used L6 myoblasts growing in 10% serum (the "proliferation" medium). To induce quiescence and promote the differentiation, the serum content was lowered to 2%. In our experimental model, L6 myoblast differentiation was induced by insulin stimulation [Pontecorvi et al., 1988]. Although in 2% serum-culture conditions the differentiation process is well controlled and the differentiation index is high, we opted for a "non-standard" method to induce L6 myogenesis since variation in HMG-CoAR levels is observed when cellular lipid composition is changed [Goldstein et al., 2006], and moreover, L6 cells are able to initiate the differentiation process even in the presence of 10% FBS (data not shown).

It has already been shown that, in our experimental model, inhibition of HMG-CoAR activity and reduction in protein levels completely prevents L6 myoblast differentiation [Martini et al., 2009]. To confirm these data we performed a time-course of insulin's effects on HMG-CoAR levels, inhibited enzyme activity using mevinolin and decreased HMG-CoAR levels by adding 25-OH cholesterol to the culture medium. As shown in Figure 1a, enzyme levels increased at 6 h, remained constant up to 16 h, and then decreased below control levels at 72 h after insulin treatment; moreover, mevinolin and 25-OH cholesterol addition completely prevented the increase of differentiation markers (Myogenin an Myosin Heavy Chain), as shown in Figure 1b.

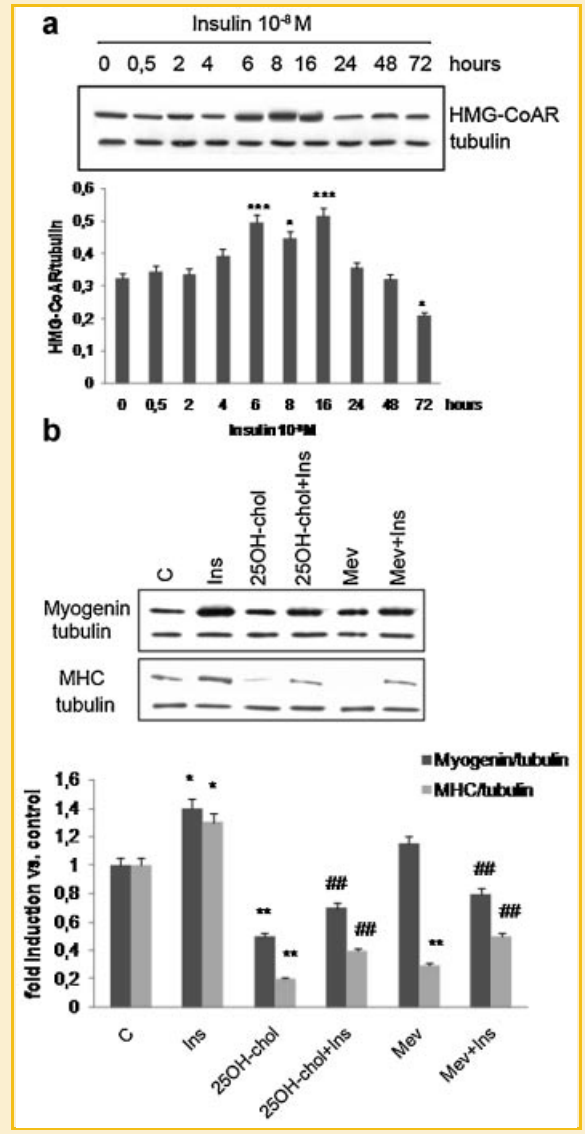

Fig. 1. HMG-CoAR time course and effects of 25-OH cholesterol and Mevinolin in insulin-treated L6 myoblasts. Panel a: The figure illustrates the time course (0–72 h) of 10^{-8} M insulin treatment on HMG-CoAR protein levels. Twenty micrograms of protein were resolved by SDS–PAGE, followed by Western blotting with HMG-CoAR antibody. The tubulin level was used as a protein loading control. For details, see the main text. Top: a typical Western blot, Bottom: densitometric analysis of three different experiments performed in duplicate. Panel b: Effects of 25-OH cholesterol and Mevinolin in insulin-induced L6 myoblast differentiation. Top: A typical Western blot, Bottom: densitometric analysis. 3 μM Mev and 25 μM 25-OH cholesterol were administrated immediately before insulin treatment. Myogenin levels were detected after 16 h post-insulin treatment while MHC levels were detected 24 h post-insulin treatment. Three different experiments were performed in duplicate. $^{*}P < 0.05$, $^{**}P < 0.001$, and $^{***}P < 0.0001$ as determined by ANOVA followed by Tukey–Kramer post-test versus C. $^{\#\#}P < 0.001$ as determined by ANOVA followed by Tukey–Kramer post test versus Ins.

INSIG mRNA AND PROTEIN LEVELS

As mentioned above, long term HMG-CoAR regulation depends on enzyme transcription and degradation. Both processes were analyzed in detail by evaluating the levels of the proteins involved in the HMG-CoAR regulatory network. Since Insig-1 is able to affect HMG-CoAR protein levels by inhibiting the expression of the gene coding the enzyme and by inducing HMG-CoAR degradation, Insig-1 mRNA and protein levels were analyzed in insulin-treated L6 cells. As illustrated in Figure 2a, Insig-1 mRNA levels increase by 8 h after

insulin stimulation and significantly decrease at 16 h, reaching control levels at 24 h. On the other hand, Insig-1 protein levels were decreased 8 h after insulin addition, increased by 16 h and diminished once again at 72 h (Fig. 2b). Comparative analysis of these results indicates that Insig-1 mRNA and protein levels change in an opposite way, in agreement with previously published data [Goldstein et al., 2006].

HMG-CoAR DEGRADATION

Insig-1 mRNA and protein level variations suggested the involvement of both degradative and transcriptional mechanisms in long term HMG-CoAR regulation. Thus, in vitro degradation assays were performed in L6 myoblasts following insulin addition.

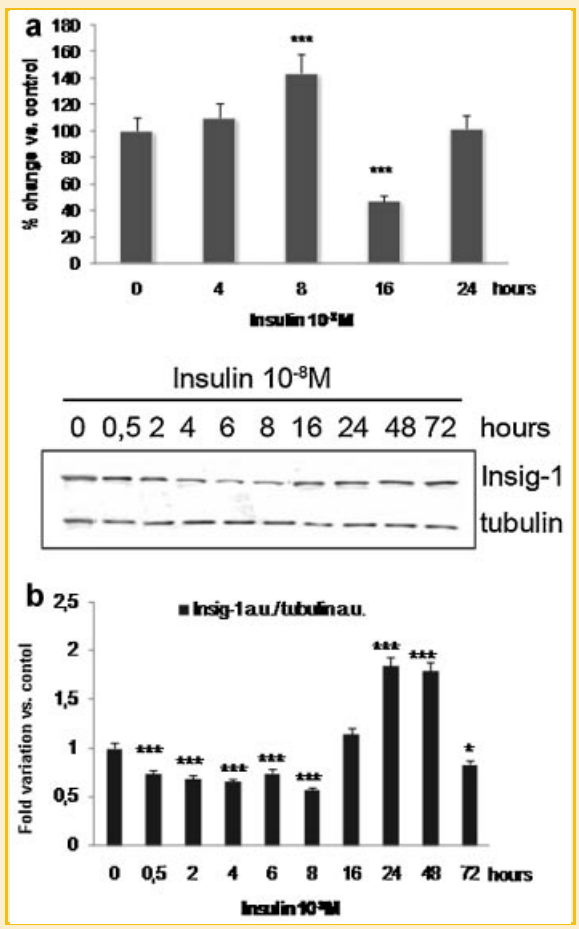

Fig. 2. Insig-1 mRNA and protein levels in insulin-induced L6 myoblast differentiation. Panel a: Relative Insig-1 mRNA levels between stimulated and control cells. qRT-PCR analysis was performed on total RNA extracted from L6 cells treated with insulin (10^{-8} M) at the times indicated. Insig-1 mRNA levels are expressed as % change versus control samples. Data are presented as the mean values ± SD of three different experiments. Panel b: Time course (0–72 h) of 10^{-8} M insulin treatment on Insig-1 protein levels. Twenty micrograms of protein were resolved by SDS–PAGE, followed by Western blotting with Insig-1 antibody. Tubulin level was used as protein loading control. For details, see the main text. Top: a typical Western blot, Bottom: densitometric analysis of three different experiments performed in duplicate. For details, see the main text. $^{**}P < 0.001$, $^{***}P < 0.0001$ as determined by ANOVA followed by Tukey–Kramer post test versus C.

The variation in HMG-CoAR protein levels from in vitro degradation assays in L6 myoblasts stimulated at different times with insulin are shown separately in Figure 3a; the data were fitted using a linear regression. The slopes obtained are shown in Figure 3b; the results indicate that the rate of HMG-CoAR degradation does not change between 16 and 48 h, declines at 4 h, and increases at 24 h after insulin stimulation.

The reduced degradation rate observed at 4 h post-stimulation could account for the precocious increase in enzyme levels, while the increase in HMG-CoAR degradation rate at 24 h after insulin addition could explain the reduction of enzyme protein levels. Thus, HMG-CoAR variations appear to be functionally related to Insig-1 protein levels.

HMG-CoAR EXPRESSION

To examine whether HMG-CoAR variations were also due to transcriptional or translational modulation, enzyme protein levels were evaluated in L6 myoblasts stimulated with insulin in presence of ACT and CHX, transcription and protein translation inhibitors, respectively. HMG-CoAR levels were checked at 6 h after insulin stimulation, when the rise in the enzyme was observed. The results shown in Figure 4 indicate that inhibition of transcription and translation by ACT and CHX, respectively, resulted in a decrease in HMG-CoAR protein levels.

The reduction in HMG-CoAR levels in presence of ACT, along with the pattern of Insig-1 expression, suggested transcriptional modulation of the enzyme; thus a time-course of SREBP-1 (HMG-CoAR transcription factor) induction by insulin was analyzed. As Figure 5 illustrates, SREBP-1 protein levels increase after 4 h, remain constant up to 8 h and then drop below control levels at 24 h.

A similar trend was observed for HMG-CoAR and Insig-1, the expression of which was reduced by SREBP-1. This suggests that SREBP-1 induces expression of HMG-CoAR followed by Insig-1 at an early stage of myogenesis.

To ascertain the involvement of transcriptional mechanisms in long term HMG-CoAR regulation, mRNA levels were measured in L6 myoblasts at 4 and 48 h after insulin treatment. Those times were chosen based on the HMG-CoAR protein levels variations previously observed. As shown in Figure 6, HMG-CoAR mRNA levels were significantly elevated at 4 h and decreased at 48 h post-stimulation, in agreement with our observed variations in HMG-CoAR protein levels.

DISCUSSION

Skeletal muscle damage is known to depend on traumatic, ischemic, pharmaceutical, toxic, metabolic, or infectious cell damage that influences the integrity of the plasma membrane (sarcolemma) and leads to the release of toxic intracellular material into systemic circulation and to muscular fiber necrosis. These pathological conditions could benefit from an enhancement in myogenesis.

Myogenesis consists of commitment and progression of myoblasts, both processes requiring the interplay of positive and negative regulatory signals. As they elongate, myoblasts align with each other, guided in this process by mutual membrane recognition.

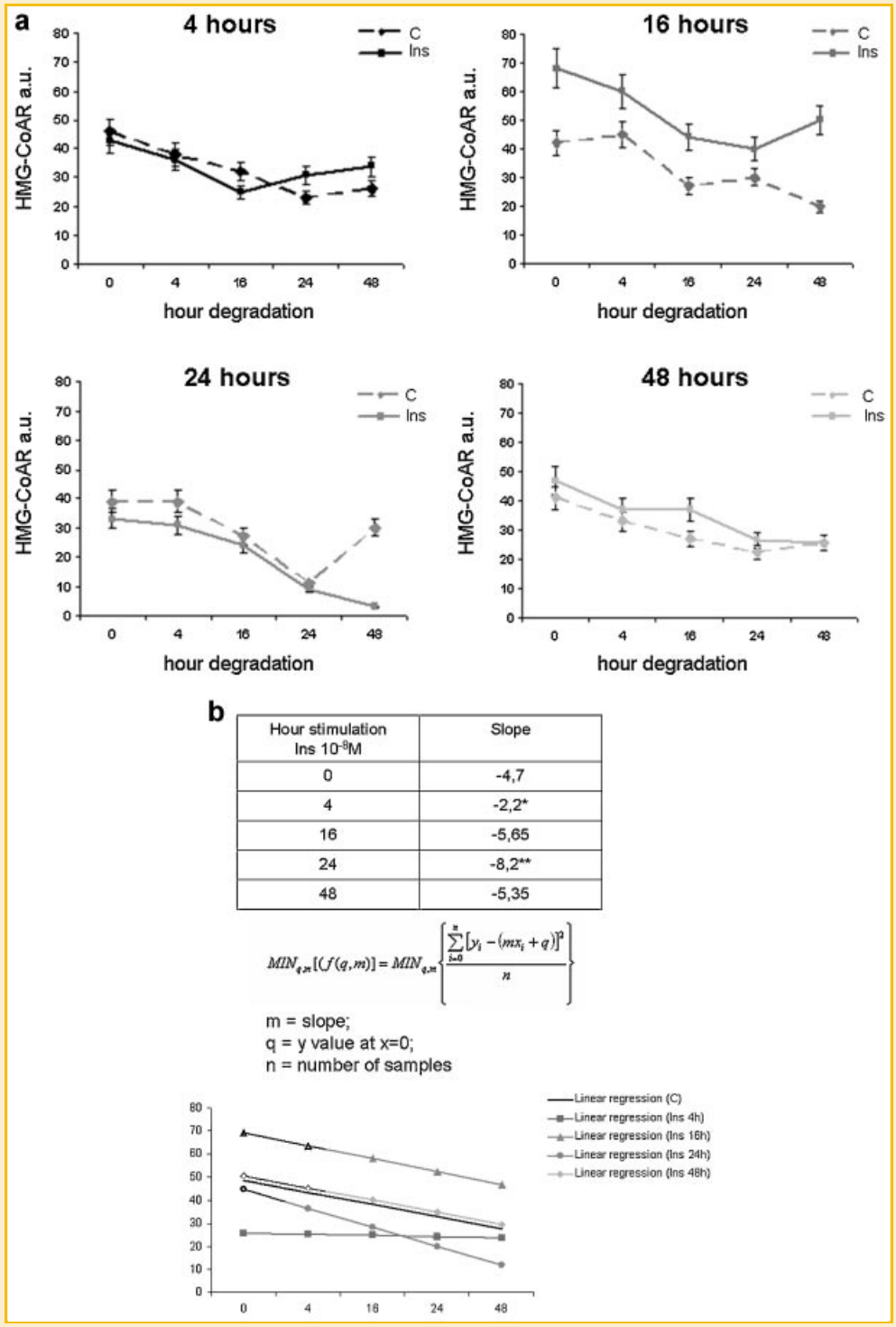

Fig. 3. HMG-CoAR degradation rate in insulin-treated L6 myoblasts. Panel a: Time courses of in vitro HMG-CoAR degradation in L6 myoblasts treated with insulin 10^{-8} M for 4, 16, 24, and 48 h. At the end of each insulin stimulation, each sample was sonicated and then incubated at 37°C in a specific buffer (detailed in the text). From these samples, lysates were collected at 4, 16, 24, and 48 h. Degradation was blocked in cold lysis buffer and HMG-CoAR levels were analyzed. HMG-CoAR protein levels were evaluated by Western blot. Twenty micrograms of protein were resolved by SDS–PAGE, followed by Western blotting with HMG-CoAR antibody. Data are presented as the mean values ± SD of three different experiments. The data were fitted using a linear regression shown in panel b.

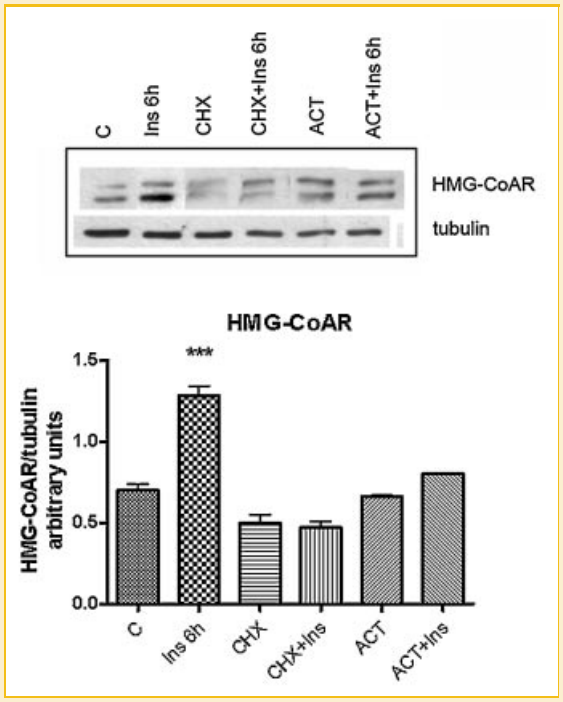

Fig. 4. Effects of actinomycin and cycloheximide in insulin-induced L6 myoblast differentiation. Top: a typical Western blot, Bottom: densitometric analysis of three different experiments performed in duplicate. cycloheximide (CHX) (10 μg/ml) and actinomycin (ACT) (1 μg/ml) were administrated to cells 60 and 30 min before insulin treatment, respectively. HMG-CoAR protein levels were detected after 6 h of insulin treatment. Twenty micrograms of protein were resolved by SDS–PAGE, followed by western blotting with HMG-CoAR antibody. Tubulin level was used as protein loading control. For details, see the main text. $^{***}P < 0.05$ as determined by ANOVA followed by Tukey–Kramer post test versus C.

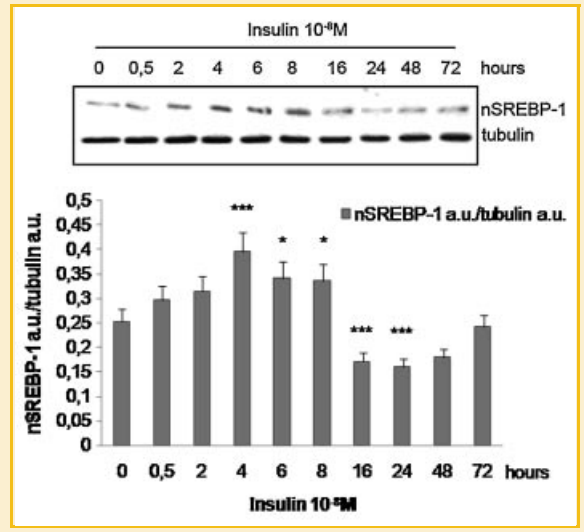

Fig. 5. SREBP-1 protein levels in insulin-induced L6 myoblast differentiation. Time course (0–72 h) of 10^{-8} M insulin treatment on SREBP-1 protein levels. Twenty micrograms of protein were resolved by SDS–PAGE, followed by Western blotting with SREBP-1 antibody. Tubulin level was used as protein loading control. For details, see the main text. Top: a typical Western blot, Bottom L densitometric analysis of three different experiments performed in duplicate. For details, see the main text. $^{*}P < 0.05$; $^{***}P < 0.0001$ as determined by ANOVA followed by Tukey–Kramer post test versus C.

Alignment is followed by cell fusion and by the formation of long, striated multinucleated myotubes [Mermelstein et al., 2007].

As recently demonstrated, HMG-CoAR appears to be up-regulated at an early stage of myogenesis and down-regulated later, moreover, inhibition of enzyme activity prevents L6 myoblast differentiation [Martini et al., 2009]. These data underline the important role for HMG-CoAR modulation in muscular differentiation. Thus, elucidation of the mechanisms responsible for HMG-CoAR modulation during muscular differentiation could be helpful in designing therapies for treatment of diseases characterized by the weakening of muscular fibers.

The data presented here demonstrate that different mechanisms are involved in long-term modulation of enzyme levels during myogenesis. At early stages of differentiation, the increase in HMG-CoAR protein levels seems to be due to transcriptional induction (parallel increase in nSREBP1 and HMG-CoAR mRNA) and to a reduction of enzyme degradation rates. These data are in agreement with the reduced amount of Insig-1, allowing both an increase in active nSREBP-1 levels and a decline in HMG-CoAR degradation rate. It is interesting to note that this induction seems dependent only on SREBP-1; in fact, no variations in SREBP-2 levels were observed (data not shown).

The subsequent reduction (24 h post insulin stimulation) in HMG-CoAR protein levels is likely due to accelerated degradation and to reduced transcription of the enzyme, both paralleled by elevated Insig-1 levels and a consequent decrease in n-SREBP-1 levels.

In the terminal stages of muscular differentiation considered in this study (48–72 h post-insulin stimulation), reduced protein levels of HMG-CoAR seem to be due solely to a decrease in transcription, as the degradation rate was unaltered. In this phase of the myogenic process, although Insig-1 levels were high, thus reducing nSREBP-1 levels, it was not able to accelerate the rate of HMG-CoAR degradation. This could be dependent on the low cholesterol levels

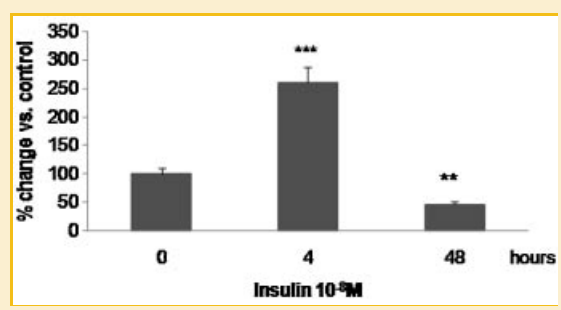

Fig. 6. HMG-CoAR mRNA levels in insulin-induced L6 myoblast differentiation. Relative levels of HMG-CoAR mRNA between stimulated and control cells. qRT-PCR analysis was performed on total RNA extracted from L6 cells treated with insulin (10^{-8} M) at the times indicated. Data are presented as the mean values ± SD of three different experiments. For details, see the main text. $^{*}P < 0.05$; $^{**}P < 0.001$ as determined by ANOVA followed by Tukey–Kramer post test versus C.

observed in this phase of the process [Martini et al., 2009] and necessary for Insig's binding to HMG-CoAR [Espenshade and Hughes, 2007].

It is interesting to note that although Insig-1 mRNA is rapidly up-regulated by insulin stimulation [Kast-Woelbern et al., 2004], the protein levels are lower than the controls in the first stage of muscular differentiation and begin to rise only at 24 h after stimulation. These observations combine to highlight the crucial role played by Insig in HMG-CoAR regulation [Sever et al., 2003], and in turn, muscular differentiation, which has already been reported [Martini et al., 2009].

Thus, the strong relationship between the modulation of HMG-CoAR activity and muscle cell differentiation previously observed [Martini et al., 2009] is consistent with the potential role of HMG-CoAR to increase the regenerative ability of damaged muscle tissue. In conclusion, our data provide the mechanisms involved in long-term HMG-CoAR regulation during myoblast differentiation and point out new targets for the design of therapeutic treatments to improve the regenerative ability of muscle tissue in degenerative myopathies and age-related muscular disorders. Modulation of Insig-1 levels could be a functionally relevant target for improving the regenerative ability of muscle cells.

ACKNOWLEDGMENTS

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