Dehydroepiandrosterone effects on the mRNA levels of peroxisome proliferator-activated receptors and their coactivators in human hepatoma HepG2 cells

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Abstract. To investigate the effect of dehydroepiandrosterone (DHEA) on intracellular mRNA levels of peroxisome proliferator-activated receptors (PPARs) and PPAR-γ coactivators (PGCs), we conducted a quantitative real-time RT-PCR study using HepG2 cells. Treatment with 100 μmol/l DHEA for 2–20 h caused a time-dependent elevation of mRNA levels in the cells. Upon 20 h, PPAR-α, -γ1, and -γ2 mRNAs and PGC-1α and -1β mRNAs increased to 157, 161, 155, 656, and 475% of control levels, respectively ($p < 0.05$ each). Treatment with actinomycin D for 2.5–8 h revealed a significant stabilization effect of DHEA on PPAR-γ1 and PGC-1α mRNAs at both 2.5 and 8 h incubation periods and a mild but significant stabilization effect on PGC-1β mRNA at the 8 h incubation period suggesting that DHEA can modulate turnover of these mRNA transcripts. Basal mRNA levels of PPAR-α and PGC-1α were significantly suppressed upon 20 h treatment with cycloheximide, while those of PPAR-γ1, -γ2, and PGC-1β were elevated. Cycloheximide also significantly reduced DHEA-induced accumulation of PPAR-α, -γ1, -γ2, and PGC-1α mRNAs, demonstrating the dependence of the DHEA action on *de novo* protein synthesis. The findings demonstrate that a supraphysiological concentration of DHEA can substantially influence gene expression of the PPAR signalling machinery at both transcriptional and posttranscriptional levels.

Key words: Dehydroepiadnrosterone — Peroxisome proliferator-activated receptor — PPAR-γ coactivator — mRNA stability — HepG2 cells

Introduction

Dehydroepiandrosterone (DHEA), the derivative of cholesterol and pregnenolone which can serve as a precursor to sex hormones, is the most abundant steroid product of the human adrenal cortex. Plasma levels of DHEA peak between 20 to 30 years of age and then decline continuously. This decline is inversely related to the occurrence of a number of civilization and/or age-related disorders (Mazat et al. 2001; Yen 2001). DHEA is expected to be a beneficial food supplement with a number of protective effects against obesity, insulin resistance, and other diseases. These expectations are mainly based on animal studies that have made use of the fact that the animal tissues can respond to exogenous DHEA. Despite much recent progress, the exact physiological, pathological, and pharmacological functions of DHEA and its mechanisms of action remain unclear (Aoki et al. 2003).

Previous studies have indicated that the dietary administration of DHEA can protect against visceral obesity and improve insulin resistance, hepatic glucose production, and hyperglycemia in rodents (Aoki et al. 2003, 2004; Hansen et al. 1997). Dose-dependent oxidation-balancing DHEA effects have been demonstrated and linked to the activation of peroxisome proliferator activator receptor-α (PPAR-α), in both rodent and human cells (Peters et al. 1996; Gallo et al. 1999; Mastrocola et al. 2003), whereas down-regulation of the expression of PPAR-γ by DHEA has been reported in rat adipocytes (Kajita et al. 2003). Furthermore, both conventional and atypical isoforms of proteinkinase C (PKC) have been implicated in various cellular DHEA effects (Ishizuka et al.

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1999; Campbell et al. 2004; Perrini et al. 2004). PPAR-α has been reported to be activated by an upstream PKC activity in both rat (Yaacob et al. 2001) and human cells of hepatic origin (Blanquart et al. 2004), and DHEA-induced PKC desensitization has been associated with reduced responsiveness of insulin-secreting cells of rat origin *in vitro* (Liu et al. 2006).

Our previous investigation demonstrated that DHEA can change expression of mRNA of novel PKC isoforms PKC-δ and PKC-ε as well as that of liver fatty acid-binding protein, a PPAR target, in human hepatoma HepG2 cells (Rypka et al. 2005). The present study was conducted to extend those observations and to evaluate DHEA effects on mRNA expression of PPAR-α, -γ1, and -γ2 isoforms in HepG2 cells. Recently, two novel PPAR-γ coactivators PGC-1α and -1β have been recognized as the key players promoting the activity of PPARs and a number of other nuclear receptors (reviewed in Finck and Kelly 2006). Their potential involvement in DHEA action has not yet been studied; therefore, we also investigated DHEA effects on their mRNA levels.

Materials and Methods

Cell culture and treatments

HepG2 cells (American Type Culture Collection, Rockville, MD, USA) were grown to about 80% confluence in Eagle's minimum essential medium (EMEM; Sigma) supplemented with 10% fetal bovine serum (FBS; Sigma), 1 mmol/l sodium pyruvate, 2 mmol/l L-glutamine, and 50 IU/ml penicillin/streptomycin in a humidified 5% CO_2/95% air atmosphere at 37°C. The medium was then switched to the same supplemented EMEM except that it lacked FBS and contained DHEA (Sigma) at 0 (controls), 1 μmol/l, or 100 μmol/l concentrations (target samples). The cells were further incubated for 2, 12, or 20 h before being assayed. Three independent experiments were performed for each time interval and DHEA concentration used. Cell cultures were routinely monitored for the absence of the major mycoplasma contaminant species using a sensitive real-time reverse transcription-polymerase chain reaction (RT-PCR) test according to Harasawa et al. (2005).

mRNA stability in the presence of actinomycin D

For the mRNA stability studies, HepG2 cells grown to about 80% confluence were treated with 100 μmol/l DHEA for 12 h, as described above. After 12 h, transcriptional inhibitor actinomycin D (4 μg/ml) was added to stop mRNA transcription for another 2.5 or 8 h. Control cells were treated with actinomycin D without DHEA for the same time intervals. Total RNA was then isolated from the cells and mRNA levels were measured by real-time RT-PCR, as described below. Three independent experiments were performed for each treatment group.

mRNA levels in the presence of cycloheximide

To examine the role of *de novo* protein synthesis in the DHEA action, HepG2 cells grown to about 80% confluence were treated with 1.4 μg/ml translation inhibitor cycloheximide alone, 100 μmol/l DHEA alone, combination of DHEA with cycloheximide (target samples), or without cycloheximide and DHEA (control). After 20 h, total RNA was isolated from the cells and mRNA levels were measured by real-time RT-PCR, as described below. Three independent experiments were performed for each treatment group.

RNA preparation and quantitative real-time RT-PCR

Total RNA was extracted from 10^6 cells using the High Pure RNA Isolation kit, and mRNA was reverse-transcribed using the Transcriptor First Strand cDNA Synthesis kit (both kits from Roche Diagnostics, Mannheim, Germany). Primers (Table 1)

Table 1. Primers used for quantitative real-time RT-PCR analyses

Target	Forward primer (5′-3′)	Reverse primer (5′-3′)	References:
PPAR-γ1	AAAGAAGCCGACACTAAACC	CTTCCATTACGGAGAGATCC	Giusti et al. (2003)
PPAR-γ2	GCGATTCCTTCACTGATAC	CTTCCATTACGGAGAGATCC	Giusti et al. (2003)
PPAR-α	ACTTATCCTGTGGTCCCCGG	CCGACAGAAAGGCACTTGTGA	Liu et al. (2004)
PGC-1α	TGTGCAACTCTCTGGAACTG	TGAGGACTTGCTGAGTGGTG	Staiger et al. (2005)
PGC-1β	GCTCTCCTCCTTCTTCCTCA	ATAGAGCGTCTCCACCATCC	Staiger et al. (2005)
Reference			
CynA	GCATACGGGTCCTGGCATCTTGTCC	ATGGTGATCTTCTTGCTGGTCTTGC	Blanquart et al. (2004)
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCATGTTGCTGTA	Kemp et al. (2003)
HPRT	GGACTGAACGTCTTGC	CTTCGTGGGGTCCTTT	–

PPAR, peroxisome proliferator-activated receptors; PGC, PPAR-γ coactivator, CynA, cyclophilin A; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPRT, hypoxanthine phosphoribosyltransferase.

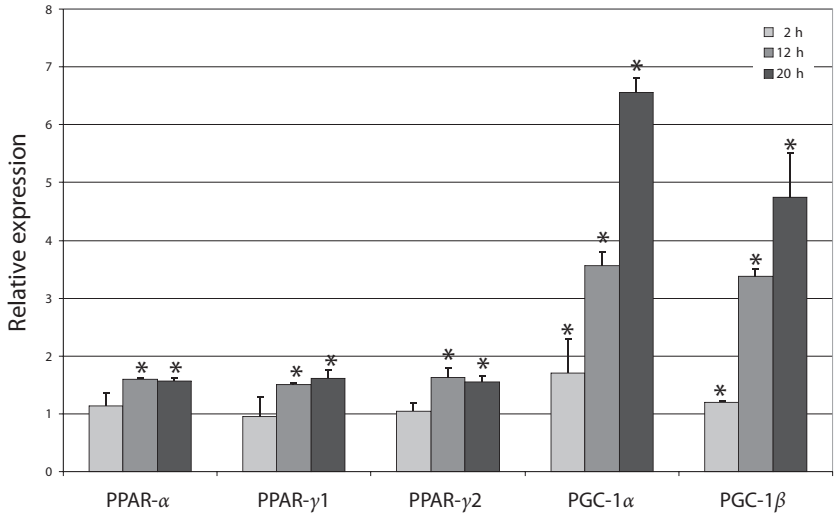

Figure 1. Effect of DHEA on mRNA levels of several PPAR-allied signalling molecules in HepG2 cells. HepG2 cells were incubated with or without (control) 100 μmol/l DHEA for 2, 12, or 20 h, and real-time RT-PCR was performed to quantify mRNA concentration levels. Mean relative mRNA expression levels of target genes were normalized by a factor derived from three reference genes and expressed as a fold-increase compared to controls that were defined as 1. Three independent experiments were performed for each treatment and each determination was executed in duplicate. Statistical analysis was done using *t*-test. * denotes significant difference from controls at $p < 0.05$.

were designed using the NCBI nucleotide database and LightCycler Probe Design software (HPRT primers) or adopted according to the data of others (Giusti et al. 2003; Kemp et al. 2003; Blanquart et al. 2004; Liu et al. 2004; Staiger et al. 2005) and were synthesized by Metabion International (Martinsried, Germany). RT-PCR was performed using the rapid thermal LightCycler system (Roche). 20 μl of a reaction mixture consisting of FastStart DNA MasterPLUS SYBR Green I (Roche), the forward and reverse primers (0.5 μmol/l each), and an aliquot of the reverse-transcribed target and control samples (2 μl) were contained in a LightCycler glass capillary. Negative controls were tested in parallel. The quality of the PCR products detected with SYBR Green I was routinely confirmed by melting curve analysis and, in initial experiments, by agarose gel electrophoresis using ChemiGenius System computerized densitometry (Syngene, Cambridge, UK).

Relative mRNA quantification and statistical evaluation

A normalization factor derived from three reference genes (Table 1) measured in the same RNA sample was used to compute the relative expression ratio. In actinomycin D and cycloheximide treatments, the treated targets were compared to the corresponding values for controls using Student's *t*-test and within groups with ANOVA and post hoc test. Three separate experiments were performed and each determination was executed in duplicate. Data represent means ± S.D. The statistical significance was set at $p < 0.05$.

Results

Relative expression ratios for mRNAs in DHEA-treated samples compared to controls (no DHEA) were estimated using quantitative real-time RT-PCR. We first focused on the effects of DHEA on PPARs. The incubation of HepG2 with 100 μmol/l DHEA for 2 h had no effect on measured PPAR isoforms. In contrast, 12 h DHEA treatment induced significant elevation in PPAR-α, -γ1, and γ2 mRNA levels in HepG2 cells (159, 150, and 162% of control levels, respectively; $p < 0.05$ for each), which remained unchanged after 20 h incubation period (157, 161, and 155% of control levels for PPAR-α, -γ1, and -γ2 mRNAs, respectively) (Fig. 1). We then asked whether DHEA might act on other major components of the PPAR machinery. Contrary to PPARs, PPAR-γ coactivators (PGC)-1α and -1β mRNAs were upregulated upon 2 h treatment with 100 μmol/l DHEA (171 and 120% of control levels, respectively; $p < 0.05$ for each). Incubation of the cells for 12 h with DHEA induced additional significant increases in the PGC-1α and -1β mRNA levels (357 and 338% of control levels, respectively; $p < 0.05$ for each) that were further elevated upon 20 h DHEA treatment (656 and 475% of control levels, respectively; $p < 0.05$ for each) (Fig. 1). No significant effects of DHEA on the levels of the above mRNA transcripts were found following exposure of the cells to 1 μmol/l DHEA (data not shown).

Considering that mRNA expression can be regulated by both transcriptional and posttranscriptional mechanisms, we

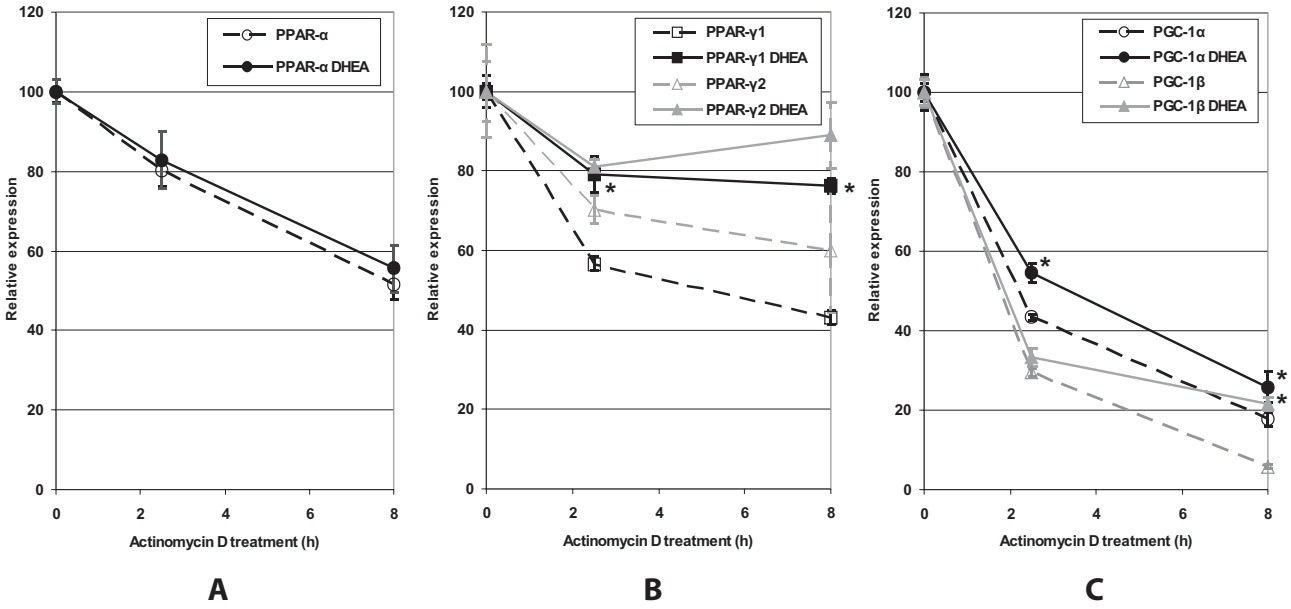

Figure 2. Effects of DHEA on mRNA stability in HepG2 cells in the presence of actinomycin D. PPAR-α mRNA (A); PPAR-γ1 and -γ2 mRNAs (B); PGC-1α and -1β mRNAs (C). Actinomycin D (4 μg/ml) was added to HepG2 cells beginning at 12 h of their incubation with 100 μmol/l DHEA and the cells were then incubated for another 2.5 and 8 h. Controls were incubated without DHEA. At the end of incubation periods the cells were harvested, mRNA analyzed by real-time RT-PCR and relative expression ratios normalized using geometric means derived from three reference genes. Signals of individual mRNAs prior to actinomycin D treatment were defined as 100%. Actinomycin D-treated mRNA levels were calculated as percentage of decay from the initial values and compared at the end of each incubation period. Three independent experiments were performed for each treatment and each determination was executed in duplicate. * denotes significant difference by *t*-test ($p < 0.05$).

then focused on whether DHEA could affect the turnover of the studied mRNAs. To address this question, the cells were incubated with DHEA for 12 h to elevate the content of mRNAs. The rate of mature mRNAs decay was then estimated upon 2.5 and 8 h treatments of the cells with actinomycin D (4 μg/ml). The decay curves of triplicate assays were plotted in Fig. 2. The slopes of PPAR-α and PPAR-γ2 mRNA decay curves did not show any significant differences between the target samples and their controls, indicating that the observed changes in PPAR-α and PPAR-γ2 mRNAs were due to DHEA-promoted mRNA synthesis rather than changes in mRNA stability (Fig. 2A,B). Furthermore, the curves showed a statistically significant slower decay of PPAR-γ1 mRNA in the cells maintained in the medium containing both DHEA and actinomycin D than in the cells incubated with actinomycin D alone. As apparent from Fig. 2B, the decrease was steeper within the first 2.5 h than in the later period. In particular, almost 80% of the initial PPAR-γ1 mRNA amount was present after 2.5 h actinomycin D treatment in combination with DHEA, while there was only 57% of the initial PPAR-γ1 mRNA amount maintained when the cells were incubated for 2.5 h with actinomycin D alone ($p < 0.05$). This decreased turnover rate in the presence of DHEA strongly suggested an involvement of a post-transcriptional process that prolonged half-life of the mRNA in HepG2 cells. The decreases in PGC-1α and PGC-1β mRNA levels (to about 43 and 30%, respectively, at 2.5 h) were even greater than those of the three PPAR mRNAs described above upon 2.5 h actinomycin D treatment (Fig. 2C). The PGC-1α mRNA decline was significantly slowed down by the presence of DHEA (decrease to only about 55% at 2.5 h; $p < 0.05$), while PGC-1β mRNA decayed equally quickly in both control and DHEA-treated cells within the first 2.5 h actinomycin D treatment. Interestingly, prolonged (8 h) incubation with actinomycin D indicated the presence of a mild but significant stabilization effect of DHEA on PGC-1β mRNA against the control (decrease to about 22% *vs.* 6% of the initial mRNA levels; $p < 0.05$) (Fig. 2C).

From the above observations it became obvious that after actinomycin D treatment, DHEA could still influence accumulation of several mRNA species suggesting participation of some posttranscriptional mechanism(s) for DHEA-induced effects. To assess further whether these effects were dependent on *de novo* protein synthesis, the cells were incubated in the presence of translational inhibitor cycloheximide (1.4 μg/ml, 20 h). From RT-PCR analyses in Fig. 3 it is apparent

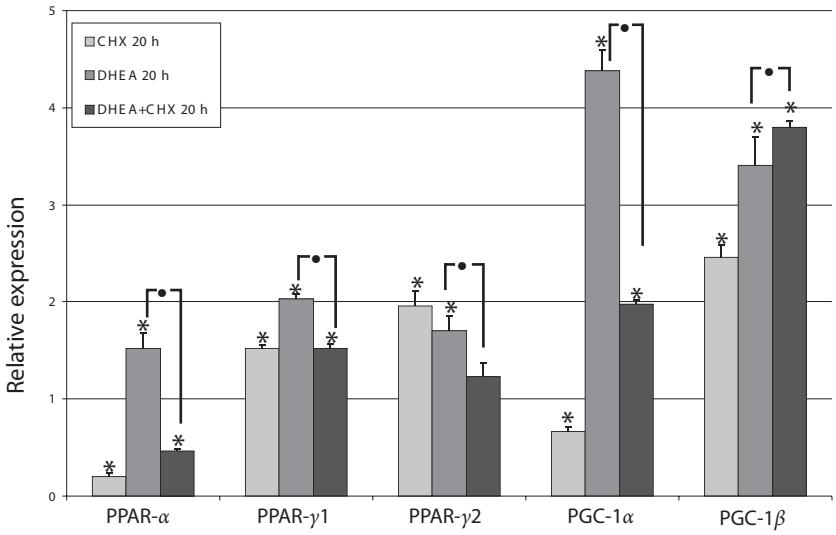

Figure 3. Effects of DHEA on mRNA stability in HepG2 cells in the presence of cycloheximide (CHX). HepG2 cells were treated for 20 h with 100 μmol/l DHEA alone, 1.4 μg/ml CHX alone, or with 100 μmol/l DHEA in the combination with 1.4 μg/ml CHX (DHEA+CHX 20 h). Control cells were incubated in the absence of both DHEA and CHX for the same period. At the end of incubation periods the cells were harvested, mRNA analyzed by real-time RT-PCR and relative expression ratios normalized using geometric means derived from three reference genes. Control mRNA levels were defined as 1, and mRNA levels of target samples were calculated as percentage of this value. Three independent experiments were performed for each treatment and each determination was executed in duplicate. * denotes significant difference ($p < 0.05$) when target and control cells were compared using *t*-test; • denotes significant difference ($p < 0.05$) for within-group variance analyzed by ANOVA with post hoc significance test.

that cycloheximide alone significantly enhanced basal levels of PPAR-γ1, -γ2, and PGC-1β mRNAs (153, 196, and 246% of control levels, respectively; *t*-test, $p < 0.05$ for each), suggesting that the transcripts were stabilized in the absence of protein synthesis. Furthermore, the DHEA-induced level of PGC-1β mRNA slightly increased when the cells were treated with DHEA in combination with cycloheximide (from 341 to 380% of control levels; ANOVA, $p < 0.05$) (Fig. 3). In contrast, cycloheximide caused a significant decline in both basal and DHEA-upregulated PPAR-α mRNA (from 100 to 20% and from 152 to 47% of control levels, respectively; $p < 0.05$ for each) and in PGC-1α mRNA (from 100 to 66% and from 439 to 198% of control levels, respectively; $p < 0.05$ for each). These findings indicated that for both basal and DHEA-induced PPAR-α and PGC-1α mRNAs accumulation, the normal protein synthesis of one or more newly synthesized proteins appeared essential (Fig. 3). However, it can be also seen from the figure that the effect of cycloheximide and that of DHEA were not simply additive.

Discussion

In this article, we have provided evidence that the treatment of HepG2 cells with a supraphysiological concentration of DHEA significantly affected mRNA expression of several members of PPAR signaling machinery, such as PPAR-α, -γ1, and -γ2 isoforms and PPAR-γ coactivators PGC-1α and -1β. Furthermore, we have demonstrated that DHEA can influence mRNA expression at both transcriptional and posttranscriptional levels.

It is now increasingly appreciated that the control of mRNA metabolism functions as a powerful mechanism for regulating gene activity (Eberhardt et al. 2007). Specifically, steroid hormones have been extensively studied as stability regulators of many mRNAs (reviewed in Ing 2005). It is believed that knowledge about molecular mechanisms of mRNA stabilities can unravel new approaches that can be exploited in therapeutic strategies.

More than a decade ago, DHEA has been recognized as a peroxisomal proliferator activating PPAR-α (Peters et al. 1996; Yen 2001). The present finding illustrated in Fig. 1 that DHEA increases PPAR-α mRNA in HepG2 is consistent with that data (Yen 2001; Depreter et al. 2002; Apostolova et al. 2005). In addition, our result obtained using actinomycin D suggests that stimulation of PPAR-α gene transcription by DHEA is the predominant factor responsible for this effect (Fig. 2A). Furthermore, the application of translational inhibitor cycloheximide has revealed that PPAR-α mRNA stability is highly dependent on a *de novo* synthesized, as yet unidentified protein(s) (Fig. 3).

Several authors have addressed the question of functional differences between the PPAR-γ1 and -γ2 variants. For in-

stance, PPAR-γ2, whose mRNA and protein are the minor PPAR-γ species in various human and rodent cells (Fajas et al. 1997, 1999; Lazar 2002; Francis et al. 2003), is thought to be a more potent inducer of adipogenesis than PPAR-γ1 (Mueller et al. 2002). Reportedly, DHEA increases PPAR-γ mRNA as well as protein (isoform not specified) in liver cells (Kajita et al. 2003; Apostolova et al. 2005), while glucocorticoids can enhance both PPAR-γ1 and PPAR-γ2 expression in adipocytes (Vidal-Puig et al. 1997). This study demonstrates for the first time that both PPAR-γ1 and -γ2 mRNAs are significantly elevated in cells upon DHEA treatment (Fig. 1). Furthermore, the present data obtained using actinomycin D and cycloheximide indicates that posttranscriptional control of mRNA stability is differently involved in regulating the PPAR-γ1 and -γ2 mRNA transcripts and that one or more DHEA- and cycloheximide-responsive *de novo* synthetized PPAR-γ mRNA stability regulator(s) can be involved in the observed effects (Figs. 2B and 3).

PGC-1α and -1β are the key PPAR coactivators. PGC-1α controls mitochondrial energy metabolism, stimulates gluconeogenesis, and interacts with PPAR-α to maintain constitutive fatty acid oxidation. PGC-1β has been also implicated in the control of cellular oxidative capacity but, in contrast to PGC-1α, PGC-1β is a poor activator of gluconeogenic genes (reviewed in Lin et al. 2005; Finck and Kelly 2006). Administration of DHEA for 12 days to mice has induced a significant decrease in the PGC-1 mRNA content (isoform not specified) in the liver (Apostolova et al. 2005). Our study demonstrates time-dependent elevation in both PGC-1α and PGC-1β mRNAs during a 2–20 h *in vitro* DHEA treatment. Both species and model variation should be taken into account to understand the dichotomy between those and our results. Notably, this study reveals important differences existing between the expression of PGC-1α mRNA and that of PGC-1β mRNA in HepG2 cells in response to a supraphysiological concentration of DHEA. The decay kinetics in Fig. 2C shows that DHEA affects PGC-1α mRNA at the posttranscriptional level through a change in mRNA stability. Consistent with this assumption is the presence of the decline of both basal and DHEA-stimulated PGC-1α mRNA levels in the presence of cycloheximide, implying that PGC-1α mRNA requires *de novo* protein synthesis to produce a positive mRNA-stability regulator in order to fully respond to DHEA signal (Fig. 3). In contrast, the change in PGC-1β mRNA appears to be mainly due to DHEA-regulated mRNA transcription, at least during the early (2.5 h) period of actinomycin D treatment. Furthermore, the occurrence of PGC-1β mRNA superinduction in the presence of cycloheximide (Fig. 3) reveals that PGC-1β mRNA is stabilized in the absence of protein synthesis, whereas PGC-1α mRNA is destabilized (Fig. 3).

In conclusion, this paper provides evidence for significant effects of a supraphysiological dose of DHEA on mRNA levels of regulatory components associated with PPAR-dependent signalling machinery. The present data suggests that modulation of transcription alone does not account for all the described mRNA changes. In parallel with other authors we speculate that certain DHEA-sensitive cellular signalling cascades trigger specific mRNA stabilization/degradation responses that can change mRNA stability and turnover (reviewed in Eberhardt et al. 2007). Our findings open way to design new experiments to unravel the processes behind these observations.

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The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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