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Evidence for differential effects of glucose and cycloheximide on mRNA levels of peroxisome proliferator-activated receptor- (PPAR-) machinery members: Superinduction of PPAR-γ1 and -γ2 mRNAs

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Quantitative real-time RT-PCR study was conducted to reveal the effects of normal (5 mmol/l) and high (30 mmol/l) glucose without or with oleate (0.3 mmol/l) on mRNA levels of peroxisome proliferator-activated receptor- (PPAR-)α, -γ1, -γ2, and peroxisome proliferator-activated receptor-γ coactivator- (PGC-)1α and -1β in commercial human hepatoma-derived HepG2 cells maintained under low-serum condition. Significant decrease in PPAR-γ1 and PGC-1α mRNA levels to about 50% was observed during the first 4 h incubation period. During the next 4 h period, both PPAR-γ1 and PGC-1α mRNAs were partly but significantly restored in high glucose batches. In this period, the presence of the transcriptional inhibitor actinomycin D revealed a significant protective effect of excess glucose on mature PPAR-γ1 and PGC-1α mRNAs. Furthermore, PPAR-γ1 and -γ2 mRNAs were differentially superinduced 1.2–2.5 fold in cells upon the administration of the translational inhibitor cycloheximide. When the cells were co-treated with the combination of cycloheximide and actinomycin D, superinduction was completely suppressed, however. Altogether, the experiments revealed, first, an unexpected protective effect of abundant glucose on PPAR-γ1 and PGC-1α mRNAs in HepG2 cells. Second, we demonstrated cycloheximide-induced, transcription-dependent upregulation of mature PPAR-γ1 and -γ2 mRNAs in HepG2 cells associated with preferential expression of the PPAR-γ2 mRNA variant. The results draw attention to as yet unexplored mechanisms involved in the control of PPAR and PGC genes.

Keywords: peroxisome proliferator-activated receptor, PPAR-γ coactivator, superinduction, mRNA stability, HepG2 cells

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INTRODUCTION

Considerable evidence has been accumulated recently showing that nutrients, such as carbohydrates, fatty acids, or amino acids can themselves control gene expression, thereby regulating cell metabolism in the varied nutritional environment (Kawaguchi *et al.*, 2002; Khan & Vanden Heuvel, 2003). Various nuclear, DNA-binding transcription factors have been identified which are able to bind nutrients and/or their intermediate metabolic products to induce adequate gene responses and metabolic actions. Of these, fatty acid-binding peroxisome proliferator-activated receptors (PPARs) have attracted extensive attention (Brown & Plutzky, 2007). PPARs are in a subfamily of nuclear receptors that include retinoic X receptor, thyroid hormone receptor, liver X receptor, and several others. Upon activation by their lipophilic ligands, PPARs interact with response elements located in promoters of their target genes and, by this action, regulate processes that are at the core of energetic metabolism. Three subtypes of PPARs, named -α, -β/δ, and -γ, have been described exhibiting differential ligand selectivity and unique expression patterns in different human tissues as well as pathologies. In particular, up to seven distinct PPAR-γ mRNAs have been identified that are produced from the same gene by alternative promoter sequences and further processed by variable mRNA splicing (Chen *et al.*, 2006; Semple *et al.*, 2006).

Another major concept of gene transcription control, complementary to that based on the transcriptional factors, has emerged recently involving the participation of transcriptional coactivators. In contrast to the transcriptional factors, the coactivators bind to and modulate the basic transcriptional machinery *via* protein–protein interactions (Tudor *et al.*, 2007). In particular, PPAR-γ coactivators (PGCs) have been implicated as master regulators transmitting environmental cues to the genes involved in the fundamental aspects of energetic metabolism. Three members of the PGC family have been identified, PGC-1α, PGC-1β, and PRC (for PGC-1-related coactivator), differentially responding to ambient impulses (Puigserver & Spiegelman, 2003; Finck & Kelly, 2006). Although PGC-1α, the best characterized representative of this family, was originally identified as a partner for PPAR-γ, it is now known to interact with and confer transcriptional specificity on many transcription factors. Relevantly, altered PGC-1α activity is associated with elevated glucose production in insulinopenic clinical states (Yoon *et al.*, 2001; Puigserver & Spiegelman, 2003). PGC-1α also plays a critical role in the regulation of heme biosynthesis (Handschin *et al.*, 2005). PGC-1β and PRC are less well studied, to date (Handschin & Spiegelman, 2006).

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Abbreviations: BSA, bovine serum albumin; CynA, cyclophilin A; DMEM, Dulbecco's modified Eagle's medium; EMEM, Eagle's minimum essential medium; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPRT, hypoxanthine phosphoribosyltransferase; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor κB; PGC, peroxisome proliferator-activated receptor-γ coactivator; PPAR, peroxisome proliferator-activated receptor; PRC, PGC-related protein; RT-PCR, reverse transcription polymerase chain reaction.

Although the basic regulation of gene expression is carried out through the amounts and activities of DNA-binding transcriptional factors, a number of data has been reported increasingly showing the control of steady-state levels of mRNA as an additional and powerful, still underappreciated mechanism for regulating gene expression in apparently all cells in human body (Ross, 1995). Steady-state mRNA levels are an outcome of mutually competing processes of mRNA formation and degradation. Depending on the cell and/or gene, mRNA half-lives range from a few minutes to days or even more, and can be regulated by both internal cellular and environmental stimuli. Very modest changes in mRNA stability and/or turnover have been described that can impact critical nodal points in cellular metabolism, thereby contributing to serious metabolic disturbances in humans. Hence, pharmacological targeting of mRNA turnover has been proposed as a potentially novel therapeutic approach (Eberhardt *et al.*, 2007).

As a part of our recent efforts to examine cellular mechanisms responsible for nutrient effects, we have described effects of glucose shortage that caused significant changes in PPAR-γ1 and PGC-1α mRNA levels in human hepatoma-derived HepG2 cells incubated under the constant ambient hormonal milieu (Bogdanova *et al.*, 2007; Poczatkova *et al.*, 2007). In the present quantitative real-time RT-PCR study we administered glucose in a supranormal concentration (30 mmol/l), without or with oleate (0.3 mmol/l), to reveal changes of PPAR-α, -γ1, -γ2, PGC-1α and -1β mRNA levels in the HepG2 *in vitro* model. Transcriptional and translational inhibitors actinomycin D and cycloheximide, respectively, were employed to learn the mechanisms of changes in mRNA levels and understand whether they can be attributed to the transcriptional or posttranscriptional mRNA metabolism in the cells. Here we report that high glucose may exert an unexpected enhancing effect on both PPAR-γ1 and PGC-1α mRNA levels in the cells. An additional and perhaps even more surprising result of the current work is the demonstration of superinduction of the PPAR-γ gene by the translational inhibitor cycloheximide.

MATERIALS AND METHODS

Materials. Eagle's minimum essential medium (EMEM), penicillin, streptomycin, sodium pyruvate, L-glutamine, trypsin, bovine serum albumin (BSA), fatty acid-free BSA, D-glucose, cycloheximide, actinomycin D, and oleic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Glucose-free Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco (Grand Island, NY, USA).

Preparation of the oleic acid-BSA complex. Fatty acid-free BSA was dissolved in EMEM medium supplemented with 1% FBS and oleic acid was added to this solution to achieve 2:1 oleic acid/BSA ratios (mol/mol). The mixture was vortexed and sonicated until optically clear and used immediately.

Cell culture and treatments. HepG2 cells (American Type Culture Collection, Rockville, MD, USA) were treated as described previously (Bogdanova *et al.*, 2007; Poczatkova *et al.*, 2007) with minor modifications. Briefly, the cells were grown to about 80% confluence in EMEM supplemented with 10% fetal bovine serum (FBS), 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. During this period the medium was replaced by fresh medium (containing all supplements) every 48 h. The medium was then switched to DMEM and supplemented with 1% FBS (w/v). BSA was adjusted in all target samples and controls to a final concentration of 1% (w/v). The experimental treatment was carried out for 4 h and 8 h periods with D-glucose at 5 or 30 mmol/l concentrations without or with the above oleic acid/BSA complex added to a final concentration of 0.3 mmol/l oleic acid. Three independent experiments were performed for each time interval and glucose and/or oleic acid concentration used and each measurement was executed in duplicate. Our cell culture was routinely monitored for the absence of the major Mycoplasma contaminant species using a real-time RT-PCR test.

The effect of actinomycin D on mRNA levels. For mRNA stability studies, HepG2 cells grown to about 80% subconfluence were incubated under zero, normal, or high glucose without or with 0.3 mmol/l oleic acid for 4 h. Then the cells were washed and incubated for an additional 4 h in the presence of the transcriptional inhibitor actinomycin D (4 μg/ml). After 4 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 time interval and glucose, oleic acid, and/or inhibitor concentration used and each measurement was executed in duplicate.

The effect of cycloheximide on mRNA levels. To examine the role of *de novo* protein synthesis under the different experimental settings, HepG2 cells grown to about 80% subconfluence were incubated for 4 h under normal (5 mmol/l) or high glucose (30 mmol/l) without or with 0.3 mmol/l oleic acid. After 4 h, HepG2 cells were further incubated without or with 1.4 μg/ml of the translational inhibitor cycloheximide for another 4 h. Another part of the cells was treated with the combination of both cycloheximide (1.4 μg/ml) and actinomycin D (4 μg/ml) for 4 h. The remaining HepG2 sets (the controls) remained untreated with the inhibitors for the same 4 h time interval. At the end of this period, total RNA was isolated from the cells and mRNA levels were measured by real-time RT-PCR. Three independent experiments were performed for each time interval and glucose, oleic acid, and/or inhibitor concentration used and each measurement was executed in duplicate.

Quantitative real-time RT-PCR for mRNA analysis. Quantitative real-time PCR was performed to measure the mRNA levels of the studied genes using the LightCycler system (Roche Diagnostics, Mannheim, Germany). 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 from Roche). Primers (Table 1; Bogdanova *et al.*, 2007; Poczatkova *et al.*, 2007) were synthesized by Metabion International (Martinsried, Germany). CynA, GAPDH, and HPRT genes were used as the reference (housekeeping) genes (Table 1). Twenty microliters of a reaction mixture consisting of FastStart DNA MasterPLUS SYBR Green I (Roche), the forward and reverse primers, and an aliquot of the reverse-transcribed samples (2 μl) were contained in a LightCycler glass capillary. Negative controls were tested in parallel.

Relative mRNA quantification and statistical evaluation. Normalization factor derived from the three reference genes (geometric mean of CynA, GAPDH, and HPRT mRNA levels) measured in the same RNA sample was used to compute the relative expression ra-

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

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	References
Target genes			
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 (housekeeping) genes			
CynA	GCATACGGGTCCTGGCATCTTGTCC	ATGGTGATCTTCTTGCTGGTCTTGC	Blanquart *et al.*, 2004
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCATGTTGCTGTA	Kemp *et al.*, 2003
HPRT	GGACTGAACGTCTTGC	CTTCGTGGGGTCCTTT	–

tio (Bustin *et al.*, 2005). The PCR data characterizing the relative mRNA expression levels in target samples were compared to the corresponding values for controls using *t*-test. The results presented in the text and the figures correspond to mean ± S.D. values. The statistical significance was set at $P < 0.05$, n = 3.

RESULTS

Effects of high glucose concentration and oleate on mRNA levels

Using quantitative real-time RT-PCR, we first compared the effects of 4 h and 8 h incubations in normal (5 mmol/l) and high (30 mmol/l) glucose on mature mRNA levels of PPAR-α, -γ1, -γ2, and PGC-1α and -1β in HepG2. In Fig. 1, relative mRNA levels of the target molecular species determined from three independent experiments at the end of the 4 h and 8 h incubation intervals were compared to their starting levels. After the first 4 h period, there was significant decrease in PPAR-γ1 mRNA to about 54% of the starting mRNA level in cells incubated in normal glucose without oleate ($P < 0.05$) (Fig. 1A). In high glucose, the PPAR-γ1 mRNA level dropped even more (44% of the starting level; $P < 0.05$) (Fig. 1A). Similarly, there was a significant decrease in PGC-1α mRNA levels in cells incubated in either normal or high glucose (44% and 28% of starting levels for normal and high glucose without oleate, respectively; $P < 0.05$) (Fig. 1B). The presence of oleate did not preclude these changes (Figs. 1A and 1B). These findings indicated that during the first 4 h incubation period, mRNAs of PPAR-γ1 and PGC-1α were significantly downregulated in the cells in low-serum medium (Figs. 1A and 1B). Cell viability was preserved during this phase, based on our routine MTT testing (not shown). Furthermore, changes in PPAR-α, -γ2, and PGC-1β mRNA levels did not reach statistical significance (cf. Figs. 2 and 3).

We next measured the relative mRNA levels in the cells after another 4 h interval. From Figs. 1A and 1B it is obvious that during this interval, excess glucose stimulated a significant 1.5- to 2.2-fold enhancement of both PPAR-γ1 and PGC-1α mRNAs (increase from 42% to 60% and from

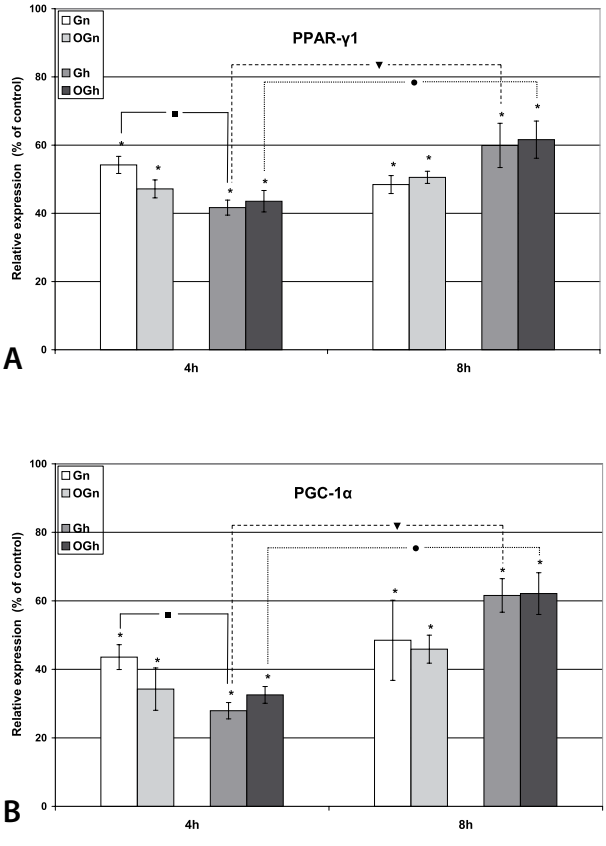

Figure 1. Effects of normal (5 mmol/l) and high (30 mmol/l) glucose and 0.3 mmol/l oleate (O) on mRNA levels of PPAR-γ1 (A) and PGC-1α (B) in HepG2 cells after 4 h and 8 h cultivation. Relative mRNA expression levels of target genes were normalized by a factor derived from three reference genes and expressed as a fold-increase compared to controls. Controls (100%) were estimated at the beginning of the experiment (0 h). Three independent experiments were performed for each treatment. Statistical analysis was done using *t*-test. Data represent mean ± S.D. (n = 3). Statistical significance was set at $P < 0.05$. *Denotes significant difference against controls; (■) denotes significant difference between normal glucose (Gn) and high glucose (Gh) settings after the first 4 h; (●) and (▼) denote significant differences between high glucose (Gh) settings measured at 4 h and those measured at 8 h. OGn, OGh, oleate with normal and high glucose, respectively.

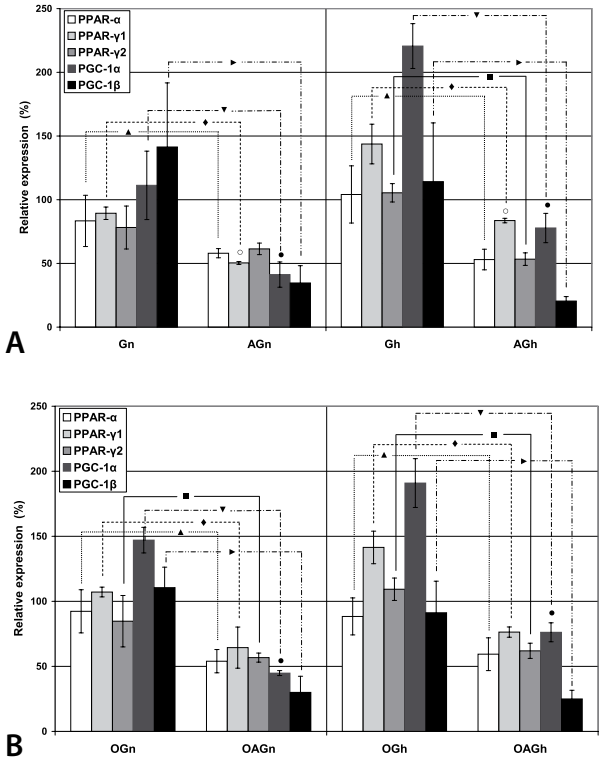

Figure 2. Effects of actinomycin D on mRNA levels of PPARs and PGCs in HepG2 cells maintained in normal (5 mmol/l; Gn) or high glucose (30 mmol/l; Gh) conditions without (A) or with (B) 0.3 mmol/l oleate.
The cells were preincubated for 4 h prior to actinomycin D administration and then treated for another 4 h with actinomycin D (4 μg/ml). mRNA was quantified by real-time RT-PCR. Relative expression ratios were normalized by a factor derived from three reference genes. Signals from individual mRNAs measured at the time of actinomycin D addition were defined as controls (100 %). Actinomycin D-treated mRNA levels were calculated as percentage of decay from the controls. Three independent experiments were performed for each treatment. Statistical analysis was done using *t*-test. Statistical significance was set at $P<0.05$. Data represent mean ±S.D. (n=3). (▲, ◆, ▼, ►) and (■) denote significant differences for samples supplemented with actinomycin D against samples without actinomycin D in the same glucose **(A)** or glucose/oleate **(B)** concentration. (●) and (○) denote significant differences between samples supplemented with actinomycin D and incubated in normal or high glucose concentration. AGn, AGh, actinomycin D with normal and high glucose, respectively. OGn, OGh, oleate with normal and high glucose, respectively. OAGn, OAGh, actinomycin D with oleate/normal glucose and oleate/high glucose, respectively.

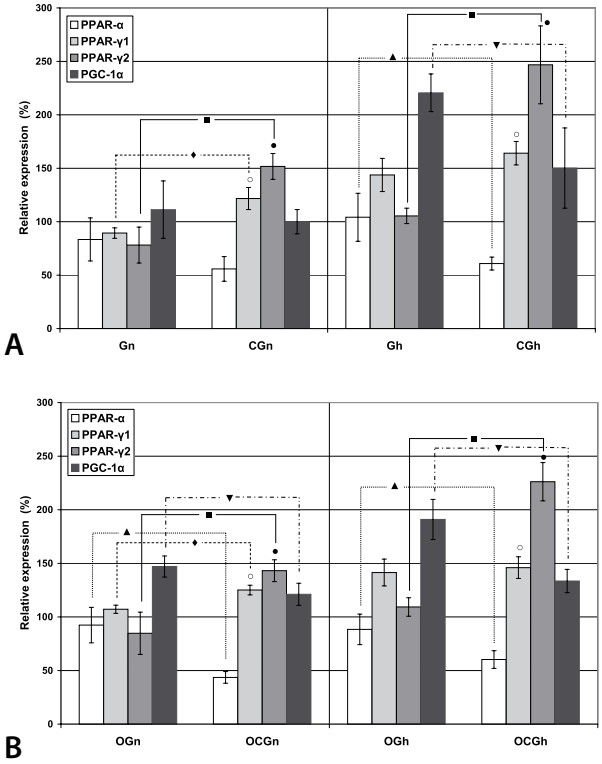

Figure 3. Effects of cycloheximide on mRNA levels of PPARs and PGCs in HepG2 cells maintained in normal (5 mmol/l; Gn) or high glucose (30 mmol/l; Gh) without (A) or with (B) 0.3 mmol/l oleate.
The cells were preincubated for 4 h prior to cycloheximide administration and then incubated for another 4 h with cycloheximide (1.4 μg/ml). mRNA was quantified by real-time RT-PCR. Relative expression ratios were normalized by a factor derived from three reference genes. Signals from individual mRNAs measured at the time of cycloheximide addition were defined as controls (100%). Cycloheximide-treated mRNA levels were calculated as percentage of the controls. Three independent experiments were performed for each treatment. Statistical analysis was done using *t*-test. Statistical significance was set at $P<0.05$. Data represent mean ±S.D. (n=3). (▲, ◆, ►) and (■) denote significant differences for samples with cycloheximide vs. samples without cycloheximide in the same glucose or glucose/oleate concentration. (●) and (○) denote significant differences in PPAR-γ1 and -γ2 mRNAs between samples supplemented with cycloheximide and incubated in high or normal glucose. CGn, CGh, cycloheximide with normal and high glucose, respectively. OGn, OGh, oleate with normal and high glucose, respectively. OCGn, OCGh, cycloheximide with oleate/normal glucose and oleate/high glucose, respectively.

28% to 62% of control levels for PPAR-γ1 and PGC-1α mRNAs, respectively; $P<0.05$ for each). This contrasted with the above apparently destabilizing effect of 30 mmol/l glucose on PGC-1α mRNA observable at the end of the initial 4 h period (Figs. 1A and 1B), indicating time-dependence of the glucose action. The presence of oleate did not interfere with the glucose effects (Figs. 1A and 1B).

Actinomycin D effects

To get further insight into molecular mechanisms of the above effects, we asked whether the nutrients affected decay rates of the mRNA transcripts. To address this question, we preincubated the cells for 4 h and then estimated the decline of mature mRNAs upon the treatment of the cells with the transcriptional inhibitor actinomycin D (4 μg/ml) for another 4 h period. The results from three independent experiments were plotted in Figs. 2A and 2B. The levels of mRNA measured at the beginning of the actinomycin D treatment were set as 100%. As expected, the levels of virtually all target mRNA species dropped significantly more upon the transcriptional blockade. However, the PPAR-γ1 and PGC-1α mRNA decay was significantly steeper upon actinomycin D treatment in the cells incubated under normal glucose than in those maintained under high glucose (50% *vs.* 84% and 41% *vs.* 78% of control levels for PPAR-γ1 and PGC-1α mRNAs, respectively; $P<0.05$ for each) (Fig 2A). This indicated that excess glucose could inhibit the decay of the two mRNAs in the course of the latter 4 h incubation interval. The presence of oleate interfered only slightly with these effects if at all (Fig. 2B).

Cycloheximide effects

As the amount of mRNA can be regulated by both transcriptional and posttranscriptional mechanisms, we

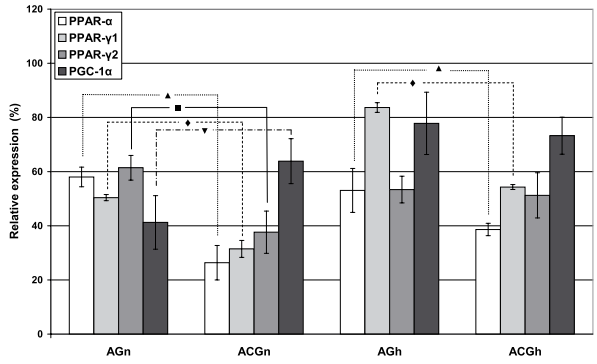

Figure 4. Comparison of the combined actinomycin D and cycloheximide treatment on mRNA levels of PPARs and PGCs in HepG2 cells with the treatment of the cells with actinomycin D alone.
The cells maintained in normal (5 mmol/l; Gn) or high glucose (30 mmol/l; Gh) were preincubated for 4 h prior to administration of inhibitors and then incubated for another 4 h in the presence of either actinomycin D alone or both inhibitors. mRNA was quantified by real-time RT-PCR. Relative expression ratios were normalized by a factor derived from three reference genes. Signals from individual mRNAs measured at the time of addition of inhibitors were defined as controls (100%). Inhibitor-treated mRNA levels were calculated as percentage of the controls. Three independent experiments were performed for each treatment. Statistical analysis was done using *t*-test. Statistical significance was set at $P<0.05$. Data represent mean ± S.D. (n = 3). (▲, ◆, ►) and (■) denote significant differences for samples supplemented with the combination of both inhibitors *vs.* samples treated with actinomycin D alone. AGn, AGh, actinomycin D with normal and high glucose, respectively. ACGn, ACGh, actinomycin D/cycloheximide with normal and high glucose, respectively.

further asked whether the mRNA levels were dependent on *de novo* protein synthesis. Therefore, after 4 h preincubation, the cells were incubated for another 4 h in the absence or the presence of the translational inhibitor cycloheximide (1.4 μg/ml). The mRNA levels at the beginning of the cycloheximide treatment were set as 100%. From Fig. 3A and 2B it is apparent that PPAR-γ1 and PPAR-γ2 mRNA levels were significantly upregulated (superinduced) in cells by the administration of cycloheximide. More specifically, PPAR-γ1 mRNA increased to about 122% of the control level in the normal-glucose batch ($P<0.05$). PPAR-γ2 mRNA was elevated to 152% and 247% of control levels ($P<0.05$ for each) in normal and high glucose settings, respectively, upon cycloheximide treatment (Fig. 3A). Notably, in 30 mmol/l glucose, cycloheximide significantly superinduced PPAR-γ2 mRNA but not PPAR-γ1 mRNA (Fig. 3A). The presence of oleate did not influence these elevations significantly (Fig. 3B). In contrast the increase in PGC-1α mRNA levels, caused by high glucose (Fig. 1B), was significantly abated by the presence of cycloheximide (150% *vs.* 221% for high glucose without oleate; $P<0.05$) (Fig. 3A). In comparison, PPAR-α mRNA levels were significantly reduced by cycloheximide in normal glucose/oleate and both high glucose settings (Figs. 3A and 3B). Finally, PGC-1β mRNA was not influenced significantly by the cycloheximide treatment (not shown). The experiments clearly demonstrated PPAR-γ1 and -γ2 mRNA species being upregulated in the presence of the translational inhibitor cycloheximide which sharply contrasted with the behavior of the other target mRNA species.

Combined actinomycin D and cycloheximide effects

Knowing that mRNA superinduction could be caused by both transcription-dependent and transcription-independent mechanisms, we next used cycloheximide along with actinomycin D. In Fig. 4 it is shown that the co-treatment of the cells with the combination of the inhibitors completely abolished the superinductive effect of cycloheximide on PPAR-γ1 and -γ2 mRNAs. Moreover, upon the co-treatment, both the PPAR-γ1 and -γ2 mRNA levels fell significantly below the levels caused by actinomycin D alone, indicating cycloheximide-assisted mRNA destabilization (31% *vs.* 50% of control values for PPAR-γ1 mRNA levels in normal glucose and 54% *vs.* 84% for PPAR-γ1 mRNA levels in high glucose; and 38% *vs.* 61% of control values for PPAR-γ2 mRNA levels for normal glucose; $P<0.05$ for each) (Fig. 4). High glucose apparently prevented this drop in PPAR-γ2 mRNA (51% *vs.* 53 % of control values) (Fig. 4). Furthermore, the decay of PPAR-α mRNA was also significantly faster when the cells were exposed to the combination of both inhibitors than that observed with actinomycin D alone (Fig. 4). Finally, our data suggested a stabilizing effect of cycloheximide on the PGC-1α mRNA level in the absence of transcription under normal-glucose conditions (Fig. 4). PGC-1β mRNA was not influenced significantly by the combined treatment (not shown).

DISCUSSION

In this study we have described the effects of abundant glucose and oleate on the expression of mature mRNAs of PPAR-α, -γ1, -γ2, PGC-1α, and -1β in Hep G2 cells incubated in a low-serum medium and in the absence of variation in ambient hormonal milieu of the cells. Quantitative real-time RT-PCR analysis with multiple reference genes was used throughout the study. Using this technique, we demonstrated an unexpected diversity in the responses of these mRNAs to supranormal (30 mmol/l) glucose and, even more surprising, to the translational inhibitor cycloheximide.

Following the initial 4 h period after the cells were challenged with low-serum medium, abundant glucose, as used in this study, had a significant positive effect on the recovery of both PPAR-γ1 and PGC-1α mRNA levels in the cells in the course of the next 4 h period. As glucose was previously shown by other authors to increase expression of the PPAR-γ gene (Panchapakesan *et al.*, 2004; Edwardsson *et al.*, 2006) as well as to influence turnover of various mRNA transcripts (Semenkovich *et al.*, 1993; Patel *et al.*, 2003), we used the transcriptional inhibitor actinomycin D to explore the basis of the observed effects. The result of the actinomycin D treatment implied the existence of (a) positive, high-glucose sensitive mechanism(s) controlling the stability of both PPAR-γ1 and PGC-1α mRNAs in HepG2 cells. Based on these results we inferred, but have not proved yet, that abundant glucose could stabilize PPAR-γ1 and PGC-1α mRNAs by as yet unknown mechanism(s). The PGC-1α mRNA stabilization by high glucose was further corroborated by the result of combined effects of actinomycin D and cycloheximide. Hyperglycemia is accepted to suppress PGC species activity through insulin (Yoon *et al.*, 2001; Puigserver & Spiegelman, 2003). Thus, it might be speculated that the increase in mRNA levels observed by us was consonant with the insulin deprivation of the cells in our experiments. Important control of PGC-1α protein has also been described to be exerted *via* the p38 MAPK pathway, however (Fan *et al.*, 2004). Therefore it is worth remembering that PGC-1α protein possesses RNA recognition and splicing domains

that enable it to participate in its own mRNA processing so that PGC-1α itself can regulate its own synthesis in a positive autoregulation loop (Monsalve *et al.*, 2000; Handschin *et al.*, 2003). Impaired protein synthesis in isolated cycloheximide treatment could therefore be expected to reduce PGC-1α mRNA production, which was actually recorded by us. However, it is premature to link the above facts into a direct causal link. We hope that the current careful analysis in our lab will shed more light on the above biphasic glucose effects on PPAR-γ1 and PGC-1α mRNAs.

Another and perhaps even more striking result of the present work was that of the evidence for the diversity of cycloheximide effects on mRNAs of the PPAR battery, and the superinduction of both PPAR-γ1 and PPAR-γ2 mRNAs that was accomplished by this translational inhibitor, in particular. We have demonstrated that cycloheximide treatment results in significant, 1.2- and 2.5-fold accumulation of PPAR-γ1 and -γ2 mRNAs, respectively, above control levels. Actinomycin D used in the combination with cycloheximide revealed that the functional transcriptional machinery is necessary for at least some critical steps involved in superinduction of the two PPAR-γ mRNA species. We therefore deduced that the predominant process responsible for the enhancement of PPAR-γ1 and -γ2 mRNA levels was cycloheximide modulation of transcription of the PPAR-γ gene. To date, the exact ways of the cycloheximide action in most cases of superinduction described in the literature remain unclear. Available data indicate that cycloheximide-caused superinduction varies across different genes as well as cell types being superinduced (Sakata *et al.*, 2007). Several hypotheses have been proposed, and it is becoming increasingly obvious that multiple mechanisms may contribute to it. First, superinduction by cycloheximide has been traditionally attributed to suppression of protein synthesis resulting in increased mRNA stability ("translation-degradation coupling" hypothesis) (Edwards & Mahadevan, 1992; Semenkovich *et al.*, 1993). Second, it has been hypothesized that cycloheximide can stimulate transcription *via* discontinuing synthesis of a putative, short-lived transcriptional repressor that, when present, could either block transcriptional initiation (a "labile repressor hypothesis") (Edwards & Mahadevan, 1992) or shut off ongoing transcription (a "post-inductive repressor mechanism") (Edwards & Mahadevan, 1992). In several cases, it has been shown that some of the superinduction effects can be triggered by cycloheximide stimulation of (an) intracellular signaling pathway(s) that could alter activity of either mRNA stabilization or transcription (co)factors by means of their posttranslational modifications (Edwards & Mahadevan, 1992). MAP kinase and/or NF-κB pathways are among the major signaling cascades studied in this context, to date (Itani *et al.*, 2003; Hershko *et al.*, 2004; Joiakim *et al.*, 2004; Tew & Hardingham, 2006). Similar to the present study, several authors have demonstrated increased levels of various mRNAs upon treatment with translational inhibitors that reflected enhanced transcriptional activity (Itani *et al.*, 2003; Hershko *et al.*, 2004). By its inducibility, indicating that PPAR-γ gene expression is not dependent on the appearance of new proteins or the continued synthesis of labile proteins, the PPAR-γ gene resembles genes of the cellular immediate early phase or the PRC gene (Edwards & Mahadevan, 1992; Vercauteren *et al.*, 2006). Additionally, we found that the levels of PPAR-γ mRNAs species decreased significantly more when the cells were co-treated with both actinomycin D and cycloheximide than did those in cells exposed to actinomycin D alone. This suggested a potential involvement of alteration of mRNA turnover by cycloheximide. Apparently, in addition to its capacity as a translational inhibitor, cycloheximide could use disparate ways to affect either transcriptional or posttranscriptional processes interrelated with functions of the PPAR-γ gene. Interestingly, PPAR-γ protein has a consensus MAPK site, similar to PGC-1α, so that its transcriptional activity can be negatively regulated upon phosphorylation by MAPK (Hu *et al.*, 1996). Future studies are required to understand the present observations in more detail.

Still another aspect seems worth mentioning related to superinduction of the PPAR-γ gene. PPAR-γ is generally accepted to be the central player in adipogenesis (Semple *et al.*, 2006). Several authors have addressed the question of functional differences between the PPAR-γ1 and -γ2 variants. For instance, PPAR-γ2, the mRNA and protein of which are the minor PPAR-γ species in various human cells, is thought to be a more potent inducer of adipogenesis than PPAR-γ1 (Chen *et al.*, 2006). Both PPAR-γ1 and -γ2 proteins are detectable in the liver, despite relatively low mRNA levels of these proteins in the cells of hepatic origin (Semple *et al.*, 2006). The present data strongly suggest that cycloheximide is able to unlatch the PPAR-γ gene and to promote its expression in favour of the PPAR-γ2 mRNA variant. It may be relevant that this cycloheximide action seems to mimic the physiological response of PPAR-γ to insulin (Edwardsson *et al.*, 2006). Intriguingly, Ketzinel and Kaempfer (1999) described a novel, as yet unexplored posttranscriptional mechanism of superinduction independent both of primary transcription and mRNA stability. When studying interleukin-2 mRNA expression, those authors showed cycloheximide facilitates splicing of a precursor RNA transcript, thereby strongly enhancing a flow into mature mRNA species (Ketzinel & Kaempfer, 1999). We believe that this finding deserves further attention, especially with respect to the fact that expression of PPAR-γ variants critically determines the overall biological effect of the PPAR-γ gene.

In summary, the present data provide evidence, first, for differential effects of supraphysiological glucose concentration on mRNA levels of the PPAR battery in HepG2 cells. Second, together with our previous findings (Bogdadova *et al.*, 2007), the results represent the first demonstration of the existence of superinduction of PPAR-γ1 and -γ2 mRNAs by cycloheximide. This is significant considering multiple biological and pathological effects of the PPAR machinery. It can be expected that further understanding of mechanisms that regulate PPARs and/or PGCs production and activity will lead to important, both clinical and pharmacological implications.

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