Construction and characterization of peroxisome proliferator-activated receptor-gamma co-activator 1 alpha (PGC-1 α over-expressing cell line derived from human hepatocyte carcinoma HepG2 cells)

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Aims. The aim was develop stable human cell line stable over-expressing transcription co-activator peroxisome proliferator-activated receptor gamma co-activator 1 α (PGC-1 α) with restored hepatospecific functions and increased expression of major xenobiotic metabolizing enzymes.

Methods. Six clones of HepG2-PGC-1 α and one control clone HepG2-pcDNA3 were isolated and analyzed for secretion of hepatospecific markers, fibrinogen, albumin and alpha1-antitrypsin. Expression levels of protein and mRNA of hepatocyte nuclear factor (HNF4 α), pregnane X receptor (PXR) and aryl hydrocarbon receptor (AhR) were determined. We measured basal and ligand inducible expression of CYP1A1 and CYP3A4.

Results. Stably transfected cell line HepG2-PGC-1 α derived from HepG2 cells over-expressing PGC-1 α displayed increased secretion of fibrinogen, but not albumin or alpha1-antitrypsin compared to parent HepG2 cells. We found increased levels of HNF4 α , PXR and AhR proteins but not their mRNAs in HepG2-PGC1 cells. Basal expression of CYP3A4 protein in HepG2-PGC-1 α cells was increased but rifampicin-inducible expression of CYP3A4 protein was lowered in comparison with parent HepG2 cells. Induction of CYP3A4 mRNA varied between 1.3 – 1.9 fold in individual clones. Expression of TCDD-inducible CYP1A1 protein was lower in HepG2-PGC-1 α cells than in parent HepG2 cells. Induction of CYP1A1 mRNA by TCDD in HepG2-PGC-1 α cells was comparable with that in parent HepG2 cells and ranged between 103 – 198 fold.

Conclusion. Stable expression of PGC-1 α in HepG2 cells restores several hepatospecific functions, such as secretion of fibrinogen, expression of HNF4 α 1 and xenoreceptors PXR and AhR. However, the expression and induction of key drug-metabolizing enzymes (CYP1A1 and CYP3A4) were not improved.

Key words: drug metabolism, cell lines, hepatocytes, cytochrome P450

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INTRODUCTION

Altered phenotype and loss of hepatocyte-specific function of human cancer cell lines derived from liver cells is probably the consequence of diminished expression of key transcriptional factors and their co-activators, such as peroxisome proliferator-activated receptor gamma co-activator (PGC-1 α). PGC-1 α was identified as the first member of the PGC-1 family co-activators¹. Its homologs PGC-1 β and PGC-1-related co-activator (PRC) display high similarity in the C-terminus, RNA recognition motif, conserved L1 motif and nuclear receptor interaction domain (L2) in N-terminus^{2,3}. PGC-1 co-activators form a complex with several HAT-containing proteins (CBP, p300, SRC-1) at their N-terminal regions or dock the protein mediator complex called TRAP/DRIP on the C-terminal region of PGC-1 α (ref.^{4,5}). PGC-1 α transcriptional activator displaces the repressor proteins and consequently increases the gene transcription⁶. PGC-1 α plays an important role in the regulation of a wide range of the nuclear receptors such as HNF4 α , glucocorticoid

receptor, PPAR α , and PPAR β and others⁷⁻⁹. Depending on the cell type, PGC-1 α is regulated in different ways. The major mechanism of PGC-1 α induction is activation of the cAMP signaling pathways through cAMP binding to CREB binding site in proximal promoter of PGC-1 α (ref.¹⁰). Another mechanism of PGC-1 α induction involves stimulation of activating transcription factor 2 (ATF2) in brown adipose tissue by cold, or calcium signaling in exercised skeletal muscles and heart^{11,12}. The expression of PGC-1 α is controlled by nutritional and hormonal signals as well as by circadian pacemakers¹³. The highest expression was found in tissues with a high density of mitochondria, i.e. heart, kidney, skeletal muscles, brown adipose tissue, liver and brain^{1,14,15}. PGC-1 α also plays an important role in adaptive thermogenesis. It is involved in glucose metabolism and maintenance of lipid and energy homeostasis. Fasting induces PGC-1 α expression in adult liver, which leads to activation of the gluconeogenic enzymes phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase through interactions with the transcription factor HNF4 α and forkhead box O1 (FOXO-1)

(ref.^{9,16,17}). Moreover, HNF4 α is a key transcriptional factor for maintenance of expression of genes encoding the hepatic xenobiotic-metabolizing enzymes P450s (ref.^{18,19}). The transactivation effect of PGC-1 α on P450 genes mediated by HNF4 α was corroborated by transfection experiments using MZ-Hep-1 cells that have low levels of HNF4 α . Co-transfection of PGC-1 α and HNF4 α increased the levels of CYP2C9, CYP1A1 and CYP1A2, whereas transfection of PGC-1 α or HNF4 α alone had no significant effect on P450s expression²⁰. Compared to primary human hepatocytes, the expression of PGC-1 α in HepG2 is greatly decreased, whereas the expression of HNF4 α is maintained²¹. Transient transfection of HepG2 cells with PGC-1 α significantly increased the expression of CYP2C9, CYP1A1 and CYP1A2 genes, and to a lesser extent the expression of CYP3A4, CYP3A5 and CYP2D6 (ref.^{20,21}).

In the present study, we describe construction and characterization of a stable cell line derived from HepG2 cells over-expressing transcription co-activator PGC-1 α . We observed increased expression of fibrinogen but not albumin or alpha1-antitrypsin in clones over-expressing PGC-1 α . The expression of HNF4 α , pregnane X receptor (PXR) and aryl hydrocarbon receptor (AhR) proteins but not their mRNAs was increased. Basal and inducible expression of xenobiotic-metabolizing enzymes CYP1A1 and CYP3A4 was not increased compared to parent HepG2 cells. In conclusion, while stable over-expression of PGC-1 α moderately improved hepatospecific functions of HepG2 cells, the resulting cell line did not display higher basal and inducible expression of CYP1A1 and CYP3A4 compared to parent HepG2 cells.

MATERIALS AND METHODS

Materials

Oligonucleotide primers used in RT-PCR reactions were from Invitrogen. LightCycler FastStart DNA Master^{PLUS} SYBR Green I and FuGENE HD transfection reagent were from Roche Diagnostic Corporation (Intes Bohemia, Czech Republic). 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was from Ultra Scientific (RI, USA). Rifampicin and DMSO were from Sigma-Aldrich (Prague, Czech Republic). Geneticin (G418) was purchased from Invitrogen (California, USA). All other chemicals were of the highest quality commercially available.

Human hepatoma HepG2 cells

Human Caucasian hepatocellular carcinoma cells HepG2 (ECACC No. 85011430) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 U/mL streptomycin, 100 μ g/mL penicillin, 4 mM L-glutamine, 1% non-essential amino acids, and 1 mM sodium pyruvate. Cells were maintained at 37 °C and 5% CO₂ in a humidified incubator.

Cytotoxicity assay

HepG2 cells were treated with G418 (400-1400 μ g/mL) for 1, 2, 3, 6 and 7 days, using 96-well plates. In parallel, the cells were treated with vehicle (DMSO; 0.1%, v/v) and Triton X-100 (1%, v/v) to assess the minimal and maximal cell damage, respectively. MTT test was performed using common protocol. Absorbance was measured spectrophotometrically at 540 nm (TECAN, Schoeller Instruments LLC). The values of IC₅₀ were calculated using data acquired from three independent cell passages.

Transfection of HepG2 cells and the selection protocol

HepG2 cells were seeded at a density of 2×10^6 cells in 60 mm culture dishes in 5 mL of DMEM culture medium and transfected with pcDNA3-neo- PGC-1 α expressing vector (2 μ g) (Dr. Ramiro Jover, Unidad de Hepatología Experimental, Centro de Investigación, Hospital La Fe, Valencia, Spain) or empty expressing vector pcDNA3-neo (2 μ g) using Fugene HD reagent. After 36 h of incubation, culture medium was replaced by selection medium supplemented with G418 (1.1 mg/mL). The medium was renewed every 3-4 days for a period of 12 weeks, until polyclonal population was selected. Cells were then transferred in 10 cm culture dishes at density of 500 – 1000 cells and cultured for an additional 5 weeks under neomycin resistance until small colonies were visible. Six colonies were subcloned into a 48-well tissue culture plate to obtain monoclonal populations. G418 resistant clones were analyzed for secretion of hepatospecific markers and expression of PGC-1 α , HNF4 α , PXR, AhR, CYP1A1 and CYP3A4. The use of GMO at the Faculty of Science, Palacky University Olomouc was approved by the Czech Ministry of Environment (ref. 91997/ENV/10).

Western blotting and protein detection

Total protein extract preparation: 3×10^6 cells were washed twice with ice-cold PBS and scraped into 1 mL of PBS. The suspension was centrifuged (4 500 RPM/5 min/4 °C) and the pellet was re-suspended in 170 μ L of ice-cold lysis buffer (10 mM Hepes pH 7.9; 10 mM KCl; 1.5 mM MgCl₂; 0.5 mM DTT; anti-protease cocktail, 0.2% (w/v) sodium dodecylsulfate). The mixture was incubated for 20 min on ice and then centrifuged (13 000 RPM/13 min/4 °C). Supernatant was collected and the protein concentration was determined by the Bradford method (Pierce, Rockford, IL.). SDS-PAGE gels (8%) were run on a Hoeffer apparatus according to the general procedure. Protein transfer onto PVDF membrane was carried out. The membrane was saturated with 5% non-fat dried milk for 2 h at room temperature. Blots were probed with primary antibodies against albumin (mouse monoclonal; sc-70340, 1.B.730), fibrinogen (mouse monoclonal; sc-65966, GMA-035), α 1-antitrypsin (mouse monoclonal; sc-73431, 13702), PGC-1 α (goat polyclonal; sc-8987, H-171), HNF4 α (rabbit polyclonal; sc-8987, H-171), PXR (mouse monoclonal; sc-48340, H-11), AhR (rabbit polyclonal; sc-5579, H-211), CYP1A1 (goat polyclonal; sc-9828, G-18), CYP3A4 (mouse monoclonal; sc-53850,

HL3) and actin (goat polyclonal; sc-1616, I-19), all purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Chemiluminescence detection was performed using horseradish peroxidase-conjugated secondary antibody and an Amersham (GE Healthcare) ECL kit. Films were scanned and the intensity of the bands was evaluated by densitometry.

mRNA determination and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

Total RNA was isolated using TRI Reagent®. cDNA was synthesized according to common protocol, using M-MLV Reverse Transcriptase (Invitrogen) and random hexamers (Amersham Biosciences). qRT-PCR was carried out on Light Cycler apparatus (Roche Diagnostic Corporation, Meylan, France). The primers and conditions for determination of CYP1A1, CYP3A4, AhR, PXR, HNF4α1 and GAPDH mRNAs were described [22]. Primers for PGC-1α were described [20]. The measurements were performed in duplicate. Gene expression was normalized per glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene. Data were processed by the delta-delta method.

Statistical Analyses

Results were expressed as means ± standard deviation.

RESULTS

Cytotoxicity of genistein

The dose of genistein was set at 1.1 mg/mL of culture medium, based on preliminary cytotoxicity experiments (data not shown).

PGC-1α expression in the stable HepG2 transfectants

HepG2 cells were transfected with pcDNA3-PGC-1α and control empty plasmid pcDNA3 carrying a *neo* selection marker conferring resistance to the antibiotic G418 (*neo* gene). We selected six G418-resistant HepG2-PGC-1α clones and one HepG2-pcDNA3 control clone. We measured the expression of PGC-1α mRNA and protein in HepG2-PGC-1α, HepG2-pcDNA3 and in parent HepG2 cells. We found no significant difference in the expression levels of PGC-1α mRNA in HepG2-PGC-1α cells compared to HepG2-pcDNA3 and parent HepG2 cells. On the other hand, we observed greatly increased

Fig. 1. PGC-1α expression in the stable G-418 resistant HepG2-PGC-1α cells. Cells were obtained as described in Materials and Methods section. **Panel A:** Representative RT-PCR of PGC-1α mRNA is shown in bar graph; western blot analyses of PGC-1α protein is above the graph. RT-PCR data are mean ± SD from duplicate measurements and are expressed as fold induction over the value from parent HepG2 cells. The data were normalized per GAPDH mRNA levels. Similar profiles, both for protein and mRNA, were observed in three independent experiments. **Panel B:** RT-PCR analysis of *Neo* resistance gene mRNA in clones of HepG2-PGC-1α cells. **Panel C:** Morphology of parent HepG2 and HepG2-PGC-1α stable cell line. The picture shows HepG2 at passage 8 and HepG2-PGC-1α stable cell line at passage 21. Numbers indicate fold change in protein expression related to the control cells HepG2 determined by densitometry.

Fig. 2. Secretion of hepatospecific markers in culture of the stable G-418 resistant HepG2-PGC1 α cells. HepG2-PGC1 α cells, HepG2-pcDNA3 cells and parent HepG2 cells were maintained in serum free medium. After 48 h, medium was removed and analyzed for secretion of hepatospecific markers. Representative western blot analyzes α 1-antitrypsin, albumin and fibrinogen proteins are showed. Similar profiles were observed in three independent experiments. Numbers indicate fold change in protein expression related to the control cells HepG2 determined by densitometry.

Fig. 3. Expression of AhR, PXR and HNF4 α in the stable G-418 resistant HepG2-PGC1 α cells. Total mRNA and total protein extracts were isolated from HepG2-PGC1 α cells, HepG2-pcDNA3 cells and parent HepG2 cells. Representative RT-PCR analyses of AhR, PXR and HNF4 α mRNAs are shown in bar graphs; western blots of AhR, PXR and HNF4 α proteins are above individual graphs. Similar profiles were obtained from three independent experiments. RT-PCR data are mean $\pm$ SD from duplicate measurements and are expressed as fold induction over the value of HepG2 parent cells. The data were normalized per GAPDH mRNA levels. Numbers indicate fold change in protein expression related to the control cells HepG2 determined by densitometry.

PGC-1 α protein level in clones 3, 4, 5 and 6 compared to control HepG2-PGC-1 α and parent HepG2 cells (Fig. 1A). We determined the mRNA expression level of *neo* gene in HepG2-PGC-1 α , HepG2-pcDNA3 and parent HepG2 cells to verify the stable integration of expressing vector (Fig. 1B). The morphology of HepG2-PGC-1 α cells was slightly different from parent HepG2 cells (Fig. 1C).

Secretion of hepatospecific markers

We analyzed HepG2-PGC-1 α clones for secretion of hepatospecific markers including α 1-antitrypsin, albumin and fibrinogen. The cells were maintained in serum-free medium for 48 h. Secretion proteins in extracellular medium were analyzed by Western immunoblotting. Secretion of fibrinogen was increased in all clones (Fig. 2). The greatest increase was observed in clones 3, 4 and 6, which correlates with the protein level of PGC-1 α and HNF4 α (Fig. 1A and 3). We found no increase in secretion of albumin and α 1-antitrypsin in any clone, as compared to HepG2-pcDNA3 and parent HepG2.

Expression of xenoreceptors and transcription factors

Next, we analyzed the expression of xenoreceptors AhR and PXR, and transcription factor HNF4 α , the most important regulators of hepatic drug-metabolizing P450s. We found increased expression of HNF4 α protein in all clones (except for clone 2) and in control HepG2-pcDNA3 cells as compared to parent HepG2 cells (Fig. 3). The greatest increase was detected in clones 3, 4, 5 and 6 and this correlates with PGC-1 α protein level. The expression level of HNF4 α mRNA was moderately decreased in HepG2-pcDNA3 control cells in comparison with parent HepG2 cells. HNF4 α mRNAs level in tested HepG2-PGC-1 α cells ranged between 0.7 fold to 1.3 fold compared to parent HepG2. The level of PXR mRNA in HepG2-pcDNA3 control cells was decreased by approx. 30% and the levels in clones 1-6 varied between 20% to 110% of the PXR level in parent HepG2 cells. On the other hand, the protein level of PXR was increased in all clones as well in control HepG2-pcDNA3 cells compared to parent HepG2 cells (Fig. 3). Expression of AhR mRNAs in the control HepG2-pcDNA3 cells was slightly increased (by approx. 40%) and in clones 1-6 varied between 90% - 160% of the AhR level in parent HepG2 cells. The AhR protein level in HepG2-pcDNA3 was not altered in comparison with parent HepG2 cells. In contrast, we found major increase in AhR protein levels in clones 3, 4 and 6 and moderate increase in clone 5 (Fig. 3), which correlates with PGC-1 α protein level (Fig. 1A).

Expression of drug-metabolizing enzymes CYP1A1 and CYP3A4

In the last series of experiments, we measured basal and ligand inducible expression of CYP1A1 and CYP3A4, the most prominent drug-metabolizing cytochromes P450. HepG2-PGC-1 α cells were treated with rifampicin (RIF; 10 μ M), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; 5 nM) and vehicle (DMSO; 0.1% V/V) for 24 h (mRNA analyzes) and 48 h (protein analyzes).

Basal expression of CYP3A4 protein in HepG2-PGC-1 α cells was markedly increased in all clones, compared to parent HepG2 and control HepG2-pcDNA3 cells. Rifampicin strongly induced CYP3A4 protein in HepG2 and HepG2-pcDNA3 cells. However, found no induction of CYP3A4 protein by rifampicin in HepG2-PGC-1 α cells, and the levels of CYP3A4 protein were nearly identical in DMSO- and RIF-treated cells. An apparent loss of CYP3A4 protein induction by RIF was due to the elevated basal levels of CYP3A4 protein in HepG2-PGC-1 α cells (Fig. 4A). Induction of CYP3A4 mRNA by RIF ranged from 1.5 to 1.7 fold in parent HepG2 and HepG2-pcDNA3 cells. Basal and RIF-inducible expression of CYP3A4 mRNAs in HepG2-PGC-1 α did not significantly differ from CYP3A4 mRNA expression in parent HepG2 cells (Fig. 4A).

The typical AhR agonist TCDD, induced CYP1A1 mRNA with similar potency in all examined cell lines, i.e., in HepG2 cells (approx. 163 fold), in HepG2-pcDNA3 cells (approx. 150 fold) cells, and in HepG2-PGC-1 α cell clones (from 65 fold to 198 fold in individual clones) (Fig. 4B). No basal level of CYP1A1 protein was found in either cell line under examination. TCDD-inducible expression of CYP1A1 protein was diminished in control cells HepG2-pcDNA3 compared to parent HepG2 cells. The level of CYP1A1 inducible protein was in all clones of HepG2-PGC-1 α cells higher than in control cells HepG2-pcDNA3, but similar to the levels in parent HepG2 cells. In clone 1 we found stronger induction of CYP1A1 protein than in parent HepG2 but this did not correlate with CYP1A1 mRNA level (Fig. 4B).

DISCUSSION

Human hepatocyte-derived cancer cell lines have many advantages such as availability, the unlimited lifespan, proliferation in culture, easy cryopreservation in comparison with primary human hepatocytes. For these properties, hepatoma cell lines would be a suitable model for drug-metabolism studies. However, lower biotransformation activity and lack of hepatospecific functions hinders wide use of cancer cell lines for metabolic studies. The loss of hepatic phenotype of cancer cells is at least partly caused by the under-expression of transcription factors or co-activators such as (PGC-1 α , SRC-1, SRC-2, c/EBP, HNF4 α) and others. PGC-1 α plays a role in adaptive thermogenesis, it is involved in activation of gluconeogenesis genes in the liver, and it stimulates genes important in mitochondrial function and oxidative metabolism. PGC-1 α activates P450 genes through co-activation of transcription factor HNF4 α which is a key factor for expression of hepatic drug-metabolizing P450s in mice, in human hepatocytes and for maintaining of hepatospecific functions¹⁹. Transient co-transfection of MZ-Hep-1 hepatocyte carcinoma cells with PGC-1 α and HNF4 α increased the expression of CYP2C9, CYP1A1 and CYP1A2 (ref.²⁰). Similarly, transient transfection of HepG2 human hepatoma cells with PGC-1 α increased

Fig. 4. Expression of CYP1A1 and CYP3A4 in the stable G-418 resistant HepG2-PGC-1 α cells. G418-resistant HepG2-PGC-1 α cells, HepG2-pcDNA3 cells and parent HepG2 cells were treated with rifampicin (RIF; 10 μ M), 2,3,7,8- tetrachlorodibenzo-*p*-dioxin (TCDD; 5 nM) and vehicle (DMSO; 0.1% V/V) for 24 h (mRNA analyzes) and 48 h (protein analyzes). Representative RT-PCR analyzes and western blots of CYP3A4 (**Panel A**) and CYP1A1 (**Panel B**) are showed. Similar profiles were obtained from three independent experiments. RT-PCR data are mean $\pm$ SD from duplicate measurements and are expressed as fold induction over vehicle-treated cells in the each cell type or clone. The data were normalized per GAPDH mRNA levels.

the expression of CYP1A1, CYP1A2, CYP2C9, CYP2D6, CYP3A4 and CYP3A5 genes²⁰. Interaction of PGC-1 α with PXR, SRC-1 and HNF4 α is essential for CYP3A4 (ref.^{23,24}). Another study reported that PGC-1 α activates transcription of CYP2A5 gene through the co-activation of HNF4 α (ref.²⁵).

In a previous study, we showed that stable over-expression of HNF4 α in HepG2 cells improved some hepato-

specific functions including secretion of plasma proteins or increased expression of xenoreceptors PXR and AhR²². Hence, we tested whether stable over-expression of PGC-1 α in HepG2 restores hepatospecific functions. In clones with increased PGC-1 α protein levels, we found strongly increased HNF4 α protein level, which is in accordance with findings of other groups^{20,23,25}. Secretion of albumin and proteins involved in the blood coagulation cascade

are hepatospecific features of the cells. HNF4 α , regulates the expression of HNF1 (ref.²⁶), which is involved in the expression of albumin and fibrinogen^{27,28}. In addition, a promoter of the α 1-antitrypsin gene contains a HNF4 α binding site²⁹. We found increased secretion of fibrinogen but not albumin and α 1-antitrypsin in all tested clones of HepG2-PGC-1 α cells. However, stable over-expression of HNF4 α in HepG2 increased the secretion of all these three plasma proteins²². Xenoreceptors AhR and PXR are the main transcriptional regulators of drug-metabolizing enzymes CYP1A1 and CYP3A4, respectively. We found that over-expression of PGC-1 α , and consequently increased expression of HNF4 α protein, positively correlated with increased levels of PXR and AhR proteins in HepG2-PGC1 α clones 3, 4, 5 and 6. However, we found the highest expression of PXR protein in clone 1 which expressed lower levels of PGC-1 α protein than clones 3, 4, 5 and 6. Dioxin-inducible expression of CYP1A1 mRNA and protein was not significantly altered compared to parent HepG2. Basal expression of CYP3A4 protein was increased in HepG2-PGC1 α cells, but rifampicin-inducible expression of CYP3A4 was consequently lost.

In conclusion, this is the first report on the construction of HepG2 cell line stably over-expressing human PGC-1 α . The new cell line displayed increased secretion of fibrinogen and higher expression of AhR, PXR and HNF4 α as compared to parent cell line. However, the expression of drug-metabolizing enzymes CYP1A1 and CYP3A4 was not restored, implying that the approach of stable over-expression of hepatospecific transcription factors only partly addressed the problem of the generation of hepatocyte-like cell lines.

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CONFLICT OF INTEREST STATEMENT

Author's conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article.

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