Biochimica et Biophysica Acta 1803 (2010) 1013–1019

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

cAMP-mediated regulation of HNF-4α depends on the level of coactivator PGC-1α

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ARTICLE INFO

Article history:
Received 26 November 2009
Received in revised form 26 May 2010
Accepted 28 May 2010
Available online 4 June 2010

Keywords:
Nuclear receptor
Coactivator
PKA
CREB
PGC-1α
HNF-4α

ABSTRACT

Hepatocyte nuclear factor-4 alpha (HNF-4α) is a member of the nuclear receptor superfamily with important roles in hepatic metabolism. Fasting induces the cAMP/protein kinase A (PKA)-signaling pathway. The mechanisms whereby cAMP regulates HNF-4α transcriptional activity are incompletely understood. We have therefore investigated the role of cAMP/PKA in regulation of HNF-4α in COS-1 cells and the hepatoma HepG2 cell line. cAMP/PKA inhibited the transcriptional activity of HNF-4α in COS-1 cells, whereas a stimulatory effect was observed in HepG2 cells. The cAMP-induced inhibition of HNF-4α in COS-1 cells was counteracted by overexpression of the nuclear receptor coactivator PGC-1α, and cAMP/PKA-dependent induction of the PGC1A gene in HepG2 cells seems to explain the cell specific differences. This was further supported by knock-down of PGC-1α in HepG2 cells, which abolished the stimulatory effect of PKA on HNF-4α transcriptional activity. Similar to the cAMP/PKA-mediated regulation of HNF-4α, overexpression of the cAMP-response element binding protein (CREB) inhibited the transcriptional activity of HNF-4α in COS-1 cells, regardless of cAMP/PKA activation and CREB phosphorylation. Moreover, activation of CREB by cAMP/PKA further stimulated HNF-4α transactivation in HepG2 cells. cAMP induced the expression of the HNF-4α target genes PCK1 and G6Pase in these cells. In conclusion, our results suggest that the level of PGC-1α determines whether the cAMP/PKA-pathway overall stimulates or inhibits HNF-4α transcriptional activation.

1. Introduction

Glucagon is a key regulator of metabolism by stimulating the cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) signaling pathway, which in turn modulates transcription of genes that control key metabolic processes. Several cAMP responsive transcription factors have been characterized [1], including the well-described element binding protein (CREB) which is phosphorylated and activated by PKA [2].

Nuclear receptors (NRs) comprise a large family of ligand-dependent transcription factors that play important roles in cell differentiation, reproduction, homeostasis and disease [3,4]. The liver-enriched nuclear receptor hepatocyte nuclear factor (HNF)-4α (NR2A1) is a highly conserved and constitutively active member of the NR family [5]. It has been shown that HNF-4α is expressed in liver, pancreas, kidney and intestine [5–7], and it binds to as many as ~12% of 13,000 examined promoters in human hepatocytes and pancreatic islets, emphasizing the versatile role of this nuclear receptor in gene regulation [8]. HNF-4α is required for embryogenesis and development [9] and plays a critical role in hepatic glucose and lipid metabolism [10–12]. Mutations in HNF-4α result in impaired insulin secretion and a monogenic form of diabetes [13]. A better understanding of the mechanisms that regulate HNF-4α-mediated transcription may lead to novel treatments for a variety of diseases [14].

The transcriptional activity of HNF-4α is modulated by posttranslational modifications, and it contains 21 serine, six threonine and seven tyrosine residues where phosphorylation may potentially alter its DNA binding activity and transactivation potential [15]. PKA and p38 mitogen-activated protein kinase mediate specific phosphorylations of HNF-4α [16,17]. The transcriptional activity of HNF-4α also depends on its interactions with different nuclear receptor coregulators, RNA polymerase II, and members of the basal transcription machinery. Nuclear receptor coactivators and corepressors are diverse molecules that modulate the gene transcription mediated by NRs [18–20]. The peroxisome proliferator-activated receptor gamma coactivator (PGC)-1α, the cAMP-response-element-binding protein/p300-interacting protein, and the members of the p160 steroid receptor coactivator (SRC) family [SRC-1, SRC-2 and SRC-3], have been reported to interact with HNF-4α [21–23]. The mechanisms controlling the activity of HNF-4α coregulators are only partly elucidated, but several studies have reported that posttranslational modifications such as sumoylation, ubiquitination, phosphorylation and acetylation elicit specific effects on their ability to activate NRs and to interact with other transcription factors [24–26].

Abbreviations: 8-CPT, 8-parachlorophenylthio; cAMP, cyclic adenosine monophosphate; CREB, cAMP-response element binding protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; G6Pase, glucose-6-phosphatase; HNF-4α, hepatocyte nuclear factor 4α; NR, nuclear receptor; PBGD, porphobilinogen deaminase; PCK1, phosphoenolpyruvate carboxykinase; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator-1alpha; PKA, protein kinase A; TBP, tata-box binding protein

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doi:10.1016/j.bbamcr.2010.05.008

The transcriptional coactivator PGC-1α regulates a wide range of processes involved in energy production and utilisation, including adaptive thermogenesis, mitochondrial biogenesis, glucose uptake and metabolism in muscle, and skeletal muscle fibre-type switching [27]. In the liver, PGC-1α is induced during fasting and stimulates hepatic *de novo* glucose synthesis (gluconeogenesis), at least in part through its stimulatory effect on HNF-4α [28,29]. Fasting has also been observed to alter the expression of bile acid transporter in hepatocytes via PGC-1α and HNF-4α [30]. Target genes of HNF-4α/PGC-1α include phosphoenolpyruvate carboxykinase (PCK1) and glucose-6-phosphatase (G6Pase), two rate-limiting enzymes in gluconeogenesis [10,31]. Moreover, the transcription factor CREB also contributes to the regulation of glucose homeostasis [29]. CREB is phosphorylated by the cAMP/PKA signaling pathway, and phosphorylated CREB stimulates PCK1 transcription via a CREB response element in the PCK1 promoter. In addition, PKA-mediated phosphorylation of CREB stimulates the expression of PGC-1α via a CREB response element in the PGC-1α promoter, and PGC-1α is one of few coactivators whose activity is modulated through regulated expression of its gene [18].

In this paper we explored the role of cAMP/PKA signaling in regulation of HNF-4α transcriptional activity, with an emphasis on PGC-1α and CREB. Using two different cell lines, the human hepatoma HepG2 cells and COS-1 cells, we demonstrate cell-specific effects of the cAMP/PKA signaling pathway, and that the cAMP/PKA-mediated regulation of HNF-4α transcriptional activity depends on the coactivator context. Our observations in HepG2 cells are in line with the model in which PKA-mediated activation of CREB enhances HNF-4α transcriptional activity via induction of the PGC-1α gene. Thus, our observations in COS-1 cells may be largely ascribed to a lack of PGC-1α induction.

2. Materials and methods

2.1. Plasmid constructs

The pGV.B2-HNF-4-tk-luc reporter plasmid for HNF-4α and the expression plasmid pcDNA3.1-HNF-4α encoding wild-type HNF-4α were kindly provided by Dr. A Fukamizu (Tsukuba, Ibaraki, Japan) and Dr. J Ladias (Boston, MA), respectively. The pGV.B2-HNF-4-tk-luc reporter plasmid is a heterologous luciferase reporter containing eight copies of a HNF-4 binding sequence from the mouse transthyretin promoter fused to a minimal TK promoter. The expression plasmid pcDNA-PGC 797 encoding wild-type PGC-1α was generously provided by Dr. D. Kelly (St. Louis, MI). The expression plasmid pCMV5-Cα encoding the catalytic subunit of PKA was a gift from Dr. G.S. McKnight (Washington, SA). The expression plasmid RSV-CREB encoding wild-type CREB was a gift from Dr. R.H. Goodman (Portland, OR). The empty vector pCMV5 was purchased from Invitrogen (Carlsbad, CA).

2.2. Cell culture and transfection experiments

African monkey kidney cells (COS-1) were cultured in Dulbecco's Modified Eagle's Medium, and human hepatoma cells (HepG2) were cultured in Eagle's Minimum Essential Medium containing non-essential amino acids and sodium pyruvate (BioWhittaker). Both media were supplemented with 10% fetal bovine serum, 2 mM L-glutamine (BioWhittaker) in 0.85% NaCl solution, 100 units of penicillin and 100 μg of streptomycin per ml. Cells were transiently transfected using SuperFect (Qiagen) according to the manufacturer's recommendations. Transfected cells were washed once with PBS and harvested 48 h after transfection. Cells were lysed in a buffer containing 25 mM TAE, pH 7.8, 2 mM DTT, 1 mM EDTA, 10% glycerol and 1% Titron-X. Luciferase assays were performed using the luciferase assay kit (BioThema AB, Sweden). All experiments were performed in triplicate.

2.3. Transfection of siRNA

siGENOME SMARTpool of PGC-1α siRNAs (Dharmacon) that specifically targets PGC-1α mRNA, and a non-specific scramble control (Scr) siRNA (5'-UGCCCAAGCACCUUAGUGC-dTdT-3', Eurogentec, Herstal, Belgium) were employed. HepG2 cells were seeded in growth medium in 24-well plates or 90 mm petri dishes 1 day before transfection. siRNAs (40 nM) were transfected twice using TransIT-TKO® Transfection Reagent (Mirus Bio LLC, WI) according to the manufacturer's protocol. The second transfection was performed 24 h after the first. For the studies of reporter gene expression, siRNA transfected cells were further transfected with plasmid DNAs using Lipofectamine 2000 (Invitrogen, CA).

2.4. Quantitative RT-PCR

Total RNA from transfected HepG2 and COS-1 cells was isolated using the RNeasy Mini Kit (Qiagen, MD) according to the manufacturer's protocol. Synthesis of cDNA from the total RNA was then performed using Transcriptor First Strand cDNA Synthesis Kit, followed by quantitative real-time RT-PCR using the LightCycler-Master SYBR Green I Kit in the LightCycler 480 rapid thermal cycler system (Roche Molecular Biochemicals). Target expression was quantified relative to a constitutive control gene, porphobilinogen deaminase (PBGD) or TATA-binding protein (TBP). The following forward (F) and reverse (R) primers were used for amplification: F (PGC-1α); 5'-AATGTGTCTCCTTCTTGTTCTT-3' and R (PGC-1α); GGTGTCTGTAGTGGCTTGA-3', F (PGC-1α in siRNA experiment); 5'-CCCATTTGAGAACAAGACTAT-3' and R (PGC-1α in siRNA experiment); 5'-GGTTATCTTGGTTGGCTTT-3'; F (PBGD); 5'-CGGAAGAAAACAGCCCAAAGA-3' and R (PBGD); 5'-TGAAGCCAGGAGGAAGCACAGT-3', and F (TBP); 5'-TTCGGAGAGTTCTGGGATTGTA-3' and R (TBP); 5'-TGGACTGTTCTTCACTCTTGGC-3'. Standards in the range of 0.01–100 pg/μl were used. Negative controls were prepared by replacing the mRNA template with PCR-grade H_2O. The RT-PCR included a 20-min reverse transcription step at 61 °C, a 5-s denaturation step at 95 °C, and then 45 cycles consisting of denaturation at 95 °C for 5 s, annealing at 54 °C for 5 s, and an extension phase at 72 °C for 18 s. Fluorescence was measured at the end of the 72 °C extension phase. The quality of the RT-PCR products was controlled by melting point curve analysis. Mean fold difference in mRNA PGC-1α expression in HepG2 cells compared to COS-1 cells was calculated using the crossing point (CP) for each sample (triplicate) and the efficiency (Eff) of the transcript, using the formula $Eff_{PGC\text{-}1\alpha}^{\Delta CP}/Eff_{PBGD}^{\Delta CP}$. For real-time PCR analyses in Fig. 6, we used LightCycler480 Probes Master (Roche) and specific Universal ProbeLibrary (UPL) probes; #3 for HNF4α, #42 for PGC-1α, #53 for PCK1 and #67 for G6Pase in duplex with UPL Human TBP Gene Assay (Roche). Gene specific primers were as following; F (HNF4α); 5'-ATTGACAACCTGTTGCAGGA-3' and R (HNF4α); 5'-CGTTGGTTCCCATATGTTCC-3', and F (PGC-1α); 5'-AAAAGCTTGACTGGCGTCAT-3' and R (PGC-1α); 3'-ACCAACCAGAGCAGCACAC-3', and F (PCK1); 5'-AAGTGCTTTGCTCTCAGGATG-3' and R (PCK1); 5'-GGGTTGGTTATACCCAGAATCA-3', and F (G6Pase); 5'-GCTGCTCATTTTCCTCATCAA-3' and R (G6Pase); 5'-TTCTGTAACAGCAATGCCTGA-3'.

2.5. Western blot analysis

Cells were lysed in a buffer containing 50 mM Tris–HCl, pH 7.5, 200 mM NaCl, 5 mM EDTA, 1% Igepal (NP40), 1 μg/ml aprotinin, 5 mM N-ethylmaleimide, 100 nM sodium orthovanate and 0.2 mM PMSF, supplemented with Complete Mini EDTA-free protease inhibitor tablets (Roche Applied Science). The cell lysates were subjected to SDS-PAGE (6 or 10%) and Western blotting. In immunoblotting, the Western membrane was first blocked with PBS (0.1% Tween) containing 5% (w/v) dried skimmed milk for 1 h. After multiple washes in PBS-Tween, the membrane was incubated with primary antibody for 1 h and secondary antibody for 30 min. For detection of