Biochemical and Biophysical Research Communications xxx (2016) 1–7

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Constitutive androstane receptor activation evokes the expression of glycolytic genes

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ARTICLE INFO

Article history:
Received 28 July 2016
Accepted 11 August 2016
Available online xxx

Keywords:
Constitutive androstane receptor
Glycolic genes
cMyc
Mitogen
TCPOBOP
Liver

ABSTRACT

It is well-known that constitutive androstane receptor (CAR) activation by 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) increases the liver-to-body weight ratio. CAR-mediated liver growth is correlated with increased expression of the pleiotropic transcription factor cMyc, which stimulates cell cycle regulatory genes and drives proliferating cells into S phase. Because glycolysis supports cell proliferation and cMyc is essential for the activation of glycolytic genes, we hypothesized that CAR-mediated up-regulation of cMyc in mouse livers might play a role in inducing the expression of glycolytic genes. The aim of the present study was to examine the effect of long-term CAR activation on glycolytic genes in a mouse model not subjected to metabolic stress. We demonstrated that long-term CAR activation by TCPOBOP increases expression of cMyc, which was correlated with reduced expression of gluconeogenic genes and up-regulation of glucose transporter, glycolytic and mitochondrial pyruvate metabolising genes. These changes in gene expression after TCPOBOP treatment were strongly correlated with changes in levels of glycolytic intermediates in mouse livers. Moreover, we demonstrated a significant positive regulatory effect of TCPOBOP-activated CAR on both mRNA and protein levels of Pkm2, a master regulator of glucose metabolism and cell proliferation. Thus, our findings provide evidence to support the conclusion that CAR activation initiates a transcriptional program that facilitates the coordinated metabolic activities required for cell proliferation.

1. Introduction

The constitutive androstane receptor (CAR), which is expressed mainly in the liver, was initially characterised as a xenosensor that regulates responses to xenochemicals. CAR mediates the up-regulation of xenobiotic/drug-metabolising enzymes, increasing the metabolic ability of the liver to protect cells from xenochemical toxicity [1]. Moreover, CAR regulates other physiologically important enzymes in the liver. For instance, CAR has been demonstrated to play a role in endogenous energy metabolism. Phosphoenolpyruvate carboxykinase (*Pck1*) and glucose-6-phosphatase (*G6pc*), key gluconeogenic genes, are repressed in response to CAR activators in a CAR-dependent manner [2–5]. CAR-mediated inhibition of β-oxidation genes is well established [6,7]. The role of CAR in the regulation of lipogenesis is more controversial [5]. CAR activation by xenobiotics also causes liver hyperplasia and hepatomegaly in the short term [8–11]. Long-term treatments with CAR activators cause liver tumours in rodents via a nongenotoxic mode of action, apparently through the induction of cell proliferation and suppression of apoptosis [9,12,13]. CAR activation is associated with the increased expression of numerous cell cycle regulators, including Cyclin D1, Mdm2, Gadd45β and cMyc [14].

As a pleiotropic transcription factor, cMyc directly stimulates cell cycle regulatory genes and drives proliferating cells into S phase for DNA replication [15]. Moreover, cMyc is essential for cell growth and proliferation through the activation of genes involved in glycolysis [16]. The proliferating cell must accumulate biomass, replicate DNA, and divide. Glucose is regarded as a major substrate for proliferating cells, providing both ATP and building blocks for macromolecular synthesis [17]. Thus, glycolysis is a platform

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http://dx.doi.org/10.1016/j.bbrc.2016.08.075
0006-291X/

supporting cell proliferation [18]. Glucose is taken into cells by transporters and trapped intracellularly by glucose phosphorylation. Hexose phosphate is further phosphorylated and split into three-carbon molecules that are converted to glycerol for lipid synthesis or sequentially transformed to pyruvate. Pyruvate is converted to acetyl-CoA, undergoes transamination to alanine, or is converted to lactate, particularly under hypoxic conditions. The glycolytic pathway is linked to the pentose phosphate pathway (PPP), which generates ribose-5-phosphate for nucleic acid synthesis and NADPH for anabolism. High rates of glycolysis are observed in proliferating cells due to the up-regulation of enzymes in glycolysis and coordinated decrease in some enzymes in gluconeogenesis.

We hypothesized that CAR-mediated up-regulation of cMyc and down-regulation of gluconeogenic genes in mouse livers might induce expression of glycolytic genes in mouse livers. The aim of the present study was to examine the effect of long-term CAR activation on glycolytic genes in a mouse model not subjected to metabolic stress. This effect was studied using a well-known, strong agonist of mouse CAR, 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP).

2. Materials and methods

2.1. Chemicals

TCPOBOP was obtained from Sigma-Aldrich (St. Louis, MO, USA), and all other analytical grade chemicals and solvents were obtained from commercial sources.

2.2. Experimental animals

Male C57BL mice (25–30 g) were supplied by the Institute of Physiology and Fundamental Medicine (Novosibirsk, Russia). Animals were acclimated for one week and allowed free access to food and water. Animals were treated i.p. with TCPOBOP in corn oil (3 mg/kg body weight) once weekly for 8 weeks. Control animals received an equal volume of corn oil. After 8 weeks, the animals were fasted and sacrificed 18 h later. Seven mice were used for each treatment group. All experimental procedures were approved by the Animal Care Committee of the Institute of Molecular Biology and Biophysics and were performed in strict accordance with National Institutes of Health guidelines.

2.3. RNA isolation, cDNA synthesis, and real-time PCR

Total RNA was isolated from livers frozen in liquid nitrogen using TRIzol (Invitrogen, USA) according to the manufacturer's protocol. First-strand cDNA synthesis was carried out with a QuantiTect Reverse Transcription Kit (Qiagen, Germany) according to the manufacturer's protocol. Gene expression levels were measured using real-time PCR with Maxima SYBR Green/ROX qPCR Master Mix (ThermoFisher Scientific, Lithuania). Specific oligonucleotide primers were used (Table 1). The expression levels of mRNA were calculated from PCR efficiency (E) and C_t and were normalised against the housekeeping gene *Mlrp*.

2.4. Preparation of whole liver extracts and western blot analysis

Preparation of whole liver extracts from mouse livers and western blot analysis were performed as previously described [19]. Sixty micrograms of proteins were separated using SDS-PAGE, transferred onto nitrocellulose membranes, and exposed to the indicated antibodies before visualization with Luminata Crescendo Western HRP Substrate (Millipore, MA, USA). Immunodetection was performed with anti-E2f1 (sc-22820), anti-cMyc (sc-788), anti-PKM2 (sc-292640, Santa Cruz Biotechnology), and anti-β-actin (Sigma-Aldrich) primary antibodies. The protein bands were analysed using a densitometric analysis program. The intensities of the signals were determined from the area under the curve of each peak.

Table 1
Oligonucleotide primers for real time PCR.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
Cyp2b10	CCCAGTGTTCCACGAGACTT	GGTGCCGACAAAGAAGAGAG
E2f1	TCTGTACCACACAGCTGCAA	GCACAGGAAAACATCAATGG
cMyc	ACGAGCACAAGCTCACCTCT	TCCAGCTCCTCCTCGAGTTA
Pck1	CCATCGGCTACATCCCTAAG	GACCTGGTCCTCCAGATACC
G6pc	CATTGTGGCTTCCTTGGTCC	GGCAGTATGGGATAAGACTG
Glut1	GGATCCCAGCAGCAAGAAGGT	CGTAGCGGTGGTTCCATGTT
Glut2	CAGAAGACAAGATCACCGGAAC	GCTGGTGTTGTGTATGCTGG
Glut3	CTTCTGTAGGACCCGAGGAAC	AGTCCTTTAGGATTGTCTCAGGT
Glut4	GTTGGTCTCGGTGCTCTTAGT	TGCCACAATGAACCAGGGAA
Gck	AAGGACAGGGACCTGGGTTCCA	TCACTGGCTGACTTGGCTTGCA
Hk2	TTCCCTTGCCAGCAGAACA	AGGTCAAACTCCTCTCGCC
Gpi1	AGCTTGTCCCCTGAGACTTC	CAACTGCAGATGGATCCTTGG
Pfkl	TGAGGATGGCTGGGAGAAC	ACCACCAGATCCTTCACGTAG
Aldoa	TGCCAGTATGTTACTGAGAAGGTC	CATGTTGGGCTTCAGCAA
Pgk1	TCACGGTGTTGCCAAAATGTC	GAACAGCAGCCTTGATCCTTTG
Pgam1	TTATGATGTCCCACCGCCTC	GAGAGACCCTCCAGATGCTT
Eno1	TTTCCTTGCTTTGCAGCGAT	GGAAGAGACCTTTTGCGGTG
Pklr	GATGCAACATGCGATTGCCC	GGTCGGTAGCGAGACAGAAG
Pkm1	GCTGTTTGAAGAGCTTGTGC	TTATAAGAGGCCTCCACGCT
Pkm2	TCGCATGCAGCACCTGATT	CCTCGAATAGCTGCAAGTGGTA
Pdha	CTGCGCTCCATGAGGAAGA	GCCGATGAAGGTCACATTTCTTA
Pdhb	CCTTGCGGCAGGCTTC	CAACTTCTTCCCCAAGCAGAA
Pdk1	CTCAACCAGCACTCCTTATTGTTC	CAAAGCCGCCTAGCGTTC
Ldha	TAATGAAGGACTTGGCGGATGA	GGAGTTCGCAGTTACACAGTAG
G6pdx	GCACTTTGTCCGGAGTGATGAA	GGTGAAAAGGGAAGATGCAGAA
Mrpl46	GGGAGCAGGCATTCCTACAG	GGTCCGGTCATTTTTTTTGTCA

2.5. Histopathology

Tissue fragments of mouse livers were fixed in 4% neutral-buffered formalin, routinely processed, and embedded in paraffin for histopathologic evaluation. Tissue sections (4–5 μm) were cut, placed onto slides, and stained with haematoxylin and eosin (HE) and Mallory's trichrome stain to visualize the tissue morphology. The analysis of histologic specimens was performed using an AxioImager A1 microscope with an AxioCam MRc camera (Carl Zeiss, Germany). Morphometry study of structural elements was conducted using a 10×/25 eyepiece with a 100×/1,3 oil lens and AxioVision (rel. 4.12.) software.

2.6. Glycolytic intermediates content

Glycolytic intermediates in mouse livers were analysed with the following Abcam (USA) products according to the manufacturer's instructions: Glucose and Sucrose Assay Kit (ab65334), Glucose-6-Phosphate Assay Kit (ab107923), Pyruvate Assay Kit (ab65342), PicoProbe Acetyl CoA Assay Kit (ab87546), PicoProbeL-Lactate Assay Kit (ab169557). Fluorescence was measured using an EnVision Multilabel Reader (PerkinElmer, USA).

2.7. Data analysis

Data are presented as the mean ± SD. Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA, USA). Comparison between the treated and control groups was performed using Student's *t*-test. A p-value < 0.05 was considered statistically significant.

Please cite this article in press as: A.A. Yarushkin, et al., Constitutive androstane receptor activation evokes the expression of glycolytic genes, Biochemical and Biophysical Research Communications (2016), http://dx.doi.org/10.1016/j.bbrc.2016.08.075