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Expression Of Gapdh, B-Actin And B-2-Microglobulin Genes Under Chemically Induced Hypoxic Conditions In Hep3b And Pc3 Cells

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Abstract

The appropriate choice of an internal standard is critical for quantitative RNA analyses. As housekeeping genes, GAPDH, β-Actin and β-2-Microglobulin are commonly employed as RNA internal standards with the assumption that their expression levels remain relatively constant in different experimental conditions. We have compared the gene expression levels of this genes under normal oxygen level and chemically induced hypoxic conditions using $CoCl_2$ in Hepotoma cell line (Hep3B) and Prostate cell line (PC3). For hypoxic conditions, Hepatoma cells (Hep3B) and prostat cells (PC3) were treated with 150 μM final consantration of $CoCl_2$ for different time intervals, namely 24, 48 and 72 hours. We used semi-quantitative RT-PCR technique to investigate mRNA level of GAPDH, β-Actin and β-2 Microglobulin in Hep3B and PC-3 cell line under chemically induced hypoxic conditions. We also analyzed β-2 Microglobulin level in HepB cell line by quantitative real-time PCR. As a result we found that the expresion levels of GAPDH and β-Actin varied widely with hypoxia but expression level of β-2-Microgloblin RNA were constant in different cell line. We concluded that hypoxic conditions can be optimized in different working conditions and in different cell type.

Key words: GAPDH, β-Aktin, β-2 Microglobulin, Hypoxia

INTRODUCTION

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-Actin and β-2-Microglobulin (β2M) are common housekeeping genes used in semi-quantitative and quantitative RNA analyses to standardize for uneven loading between samples. Ideally, internal RNA controls should be expressed constantly independent of experimental conditions. Matched loading based on these internal controls is critical for quantitative comparisons of gene expression among different tissue types, varying developmental stages, and experimentally treated cells. However, no one single housekeeping gene always manifests stable expression levels under all of these experimental conditions. Therefore, it is necessary to characterize the suitability of various housekeeping genes to serve as internal RNA controls under particular experimental conditions where transcription effects are being tested [1].

Glyceraldehyde 3-phosphate dehydrogenase is an enzyme of ~37kDa that catalyzes the sixth step of glycolysis and thus serves to break down glucose for energy and carbon molecules. In addition to this long established metabolic function, GAPDH has recently been implicated in several non-metabolic processes, including transcription activation [2, 3], initiation of apoptosis [4, 5] and ER to Golgi vesicle shuttling [6]. Another common internal control, β-actin, is one of six different actin isoforms which have been identified in humans. Actins are highly conserved proteins that are involved in cell motility, structure and integrity. β-2 microglobulin also known as β2M is a component of MHC class I molecules, which are present on all nucleated cells.

Hypoxia elicits a wide range of adaptive responses. In response to hypoxia, cells adapt by changing the transcription of genes involved in angiogenesis, erythrocyte production, cell survival and metabolism. Most of genes that are activated during hypoxia are critically regulated by hypoxia-inducible factor-1 (HIF-1α) and -2 (HIF-2). HIF is composed of an oxygen-regulated HIF-α subunit (HIF-1α and HLF) and the constitutively expressed Arnt (or HIF-1β). In normoxia, HIF-α subunits are hydroxylated by HIF prolyl hydroxylases (PHDs) at two conserved proline residues present in the oxygen-dependent degradation domain, recognized by the von Hippel-Lindau protein, ubiquitinated and degraded by the 26S proteasome [2]. The turnover of HIF-1α protein in normoxia is very rapid and it stabilizes and accumulates at hypoxia due to lack of oxygen. The accumulated HIF-1α translocates into the nucleus, dimerizes with Arnt, binds to the specific hypoxia response elements (HREs) on DNA and activates transcription by recruiting CBP/p300 transcriptional coactivators. It is estimated that 2.6% of total genes are regulated by the activation of HIF-1 [7].

Some studies reported that several genes normally used as reference genes had varying expression levels in hypoxic conditions. Some investigators found that GAPDH appears particularly unfavorable for this purpose either in hypoxia or other experimental conditions that upregulate HIF-1α [1]. However, GAPDH was reported an optimal choice of a housekeeping gene and/or loading control to determine the expression of hypoxia induced genes at least in glioblastoma.

Other contradictory finding is that GAPDH is overexpressed in LNCap, ATII, SiHa, MBEC4, EC4 and Rabits skeletal muscle cells under hypoxic conditions [8]. These findings conclusively suggested that the expression of GAPDH or any housekeeping genes could be different each cell type and physiologic conditions.

In mammalian systems, cobalt chloride ($CoCl_2$) has been used as a chemical agent that reportedly induces a biochemical and molecular response similar to that observed under low-oxygen conditions [9-14]. $CoCl_2$ binds to prolin hydroxilase and inhibit them thus HIF-1α reach to low oxygen level and effect genes which are related with hypoxia [7]. Although there is plenty of studies related with hypoxia and internal controls in the literature but there is not any study about chemically induced hypoxia and internal controls in hepatoma cells (Hep3B) and prostate cells (PC-3). The purpose of this study was to select a set of reference genes, which are suitable as internal standards in hepatoma model (Hep3B) and prostate model (PC-3) cell lines under $CoCl_2$ induced hypoxic condition.

MATERIALS AND METHODS

Materials

Human hepatoma cell line (Hep3B) was obtained from Dr. Dipak Ramji, University of Cardiff, UK and human prostat cancer cell line (PC3) were obtained from Dr. Kemal Sami Korkmaz, Ege University- EBILTEM. All tissue culture reagent are purchased from Invitrogen. Molecular biology reagents are from Fermentas.

Cell Culture and hypoxia treatment

Hep3B and PC3 cells were cultured with DMEM High Modified Medium, 10 % fetal calf serum solution and Cell cultures were seeded at 2000000 cells / 25cm^2 flask and were grown in a fully humidified incubator with 5% CO_2 at 37°C in culture flasks. Cell viability controlled by trypan blue exclusion. The cells were passaged (2 000.000 cells per flask) into 25cm^2 in 5mL medium. Cells were incubated overnight to allow cells to attach into wells. A chemical mimetic of hypoxia, $CoCl_2$, was used to cause hypoxic response of cells. For hypoxic conditions Hep3B and PC3 cells were treated with 150 µM final concentration of $CoCl_2$ for different time intervals, namely 24, 48 and 72 hours.

Isolation of total RNA from cell lines and preparation of cDNA

Total RNA from tumor cells was isolated with the RNase Easy isolation kit (Qiagene) following the manufacturer's instructions. 1000 ng of total RNA from cells was transcribed at 42°C for 1 h in a 20 µl reaction mixture using 200 U RevertAid™ M-MuLV Reverse Transcriptase (RT), oligo(dT)18 primer and 40 U Ribonuclease inhibitor.

Semiquantitative-Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed using primers designed using published information on GAPDH, β-actin and H-β-2-Microglobulin mRNA sequences in GenBank. Accession numbers of each genes were indicated in table 1 For PCR-reactions primers were designed in flanking exons with IDT software (available online (http://www.idtdna.com/analyzer/Applications /OligoAnalyzer). All primer features were indicated in table 1. The cycle number optimization were carried out for GAPDH, β-Actin and β-2-Microglobulin PCR reactions was performed with 18 cycle for GAPDH and β-actin, 19 cycle for H-β-2-Microglobulin. The reactions were carried out in duplicates. The PCR protocol consisted of an initial step at 95°C (5 min) followed by 18-30 cycles of 30 seconds at 95°C for DNA denaturation and 30 seconds of annealing at 52-60°C and elongation step at 72^{0}C (10 min) was carried out. The PCR products were separated on 2% agarose gels and visualized by ethidium bromide staining.

Quantitative Reverse Transcription-Polymerase Chain Reaction (Real Time PCR)

For the analyses of β-2-Microglobulin Quantitative PCR was performed on a Bioneer real-time PCR system (Bioneer, Exicycler™ 96 Real-Time Quantitative Thermal Block). The reactions were carried out in duplicates using the SYBR Green PCR mix (Bioneer), using the manufacturer's instructions. The final reaction volume was 20 µL, and 5 ng/ µL of primers and 1 µL of cDNA were used in each reaction. The PCR protocol consisted of an initial step at 95°C (3 min) followed by 35 cycles of 30 seconds at 95°C for DNA denaturation and 30 seconds of annealing and elongation. The annealing step was carried out at 58°C and elongation step was carried out at 72^{0}C. To confirm product specificity, a melting curve analysis was performed after each amplification.

Table 1. Housekeeping genes and their primer's information used in this study

β-2 Mikroglobulin				
Forward	**Reverse**	**Accession No**	**Product size**	**Tm**
5'TTTCTGGCCTGGAGGCTATC '3	5'CATGTCTCCATCCCACTTAACT '3	NM_004048	314 bp	60^{0}C
GAPDH				
Forward	**Reverse**	**Accession No**	**Product size**	**Tm**
5'CCCTTCATTGACCTCAACTACATGG '3	5'AGTCTTCTGGGTGGCAGTGATGG '3	NM_002046.3	455 bp	55 ^{0}C
β-Actin				
Forward	**Reverse**	**Accession No**	**Product size**	**Tm**
5'TCCCTGGAGAAGAGCTACGA '3	5'AAGAAAGGGTGTAACGCAAC '3	NM_001101.3	461 bp	50 ^{0}C

RESULTS

To explore whether expression of common housekeeping genes is modulated by hypoxia, we compared the expression level of three common housekeeping genes in Hep3B and PC3 cells cultured under normoxic or hypoxic conditions. We used semi-quantitative RT-PCR technique to investigate mRNA level of GAPDH, β-Actin and β-2 Microglobulin in Hep3B and PC-3 cell line under chemically induced hypoxic conditions. After incubation of $CoCl_2$ total RNA was collected from cells in different time namely 24, 48 and 72 hours and RT-PCR technique was used. As a control cells in normal oxygen conditions of each parameters were included in experiments. $CoCl_2$ induced hypoxia level was confirmed by mRNA analysis of HIF1-α level. HIF1-α level was increased in hypoxic conditions in $CoCl_2$ treated cells (Data not shown). RT-PCR results shows that the mRNA level of GAPDH was induced both in Hep3B and PC-3 cell lines after $CoCl_2$ incubation (Figure 2a and 2b). However, induction of GAPDH mRNA was time dependent in Hep3B cell line but not in PC3 cell line. Interestingly, the mRNA level of β-Actin was decreased in time dependent after induction of $CoCl_2$ in Hep3B cell line but we didn't see any drastic differences between normal and hypoxic conditions in PC-3 cell line (Figure 2a and 2b). Whereas, $CoCl_2$-induced hypoxic conditions affected minimally, β-2 Microglobulin mRNA level (Figure 3a and b). For the confirmation of these results we also analyzed β-2 Microglobulin level in HepB cell line by quantitative real-time PCR (Figure 4). Quantitative real-time PCR studies confirmed that β-2-Microglobulin level is not affected under hypoxic conditions and can be used as an internal control in expression studies in chemically induced hypoxic conditions.

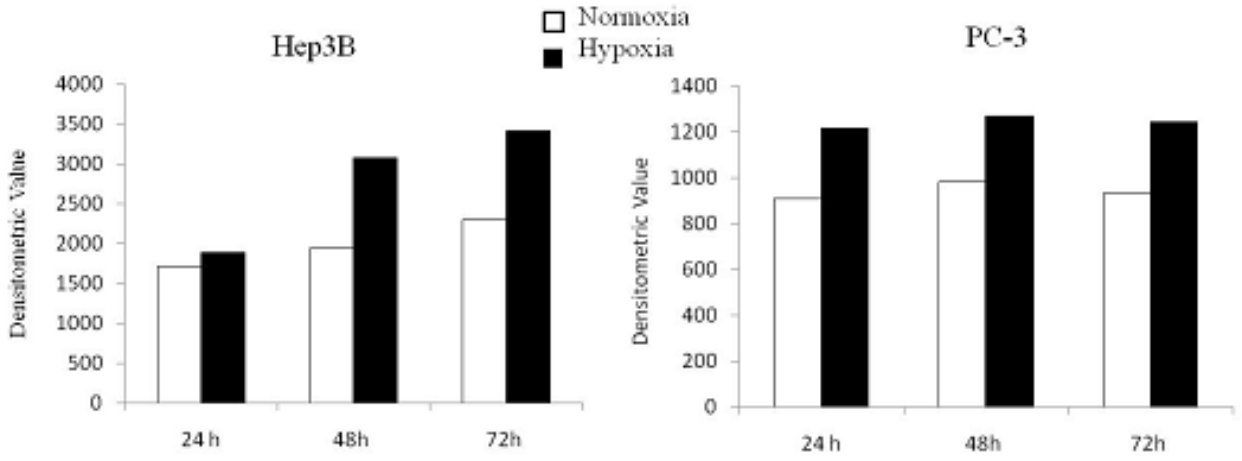

Figure 1. (a) Expression of GAPDH in Hep3B cell line in normal and hypoxic conditions
(b) Expression of GAPDH in PC-3 cell line in normal and hypoxic conditions (RT-PCR)

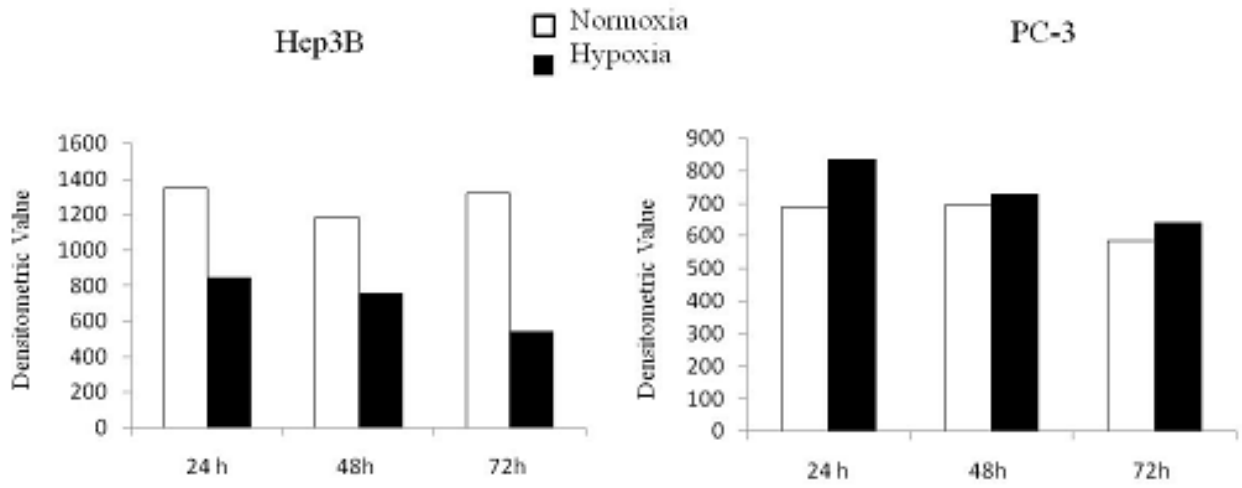

Figure 2. (a) Expression of β-actin in Hep3B cell line in normal and hypoxic conditions
(b) Expression of β-actin in PC-3 cell line in normal and hypoxic conditions (RT-PCR).

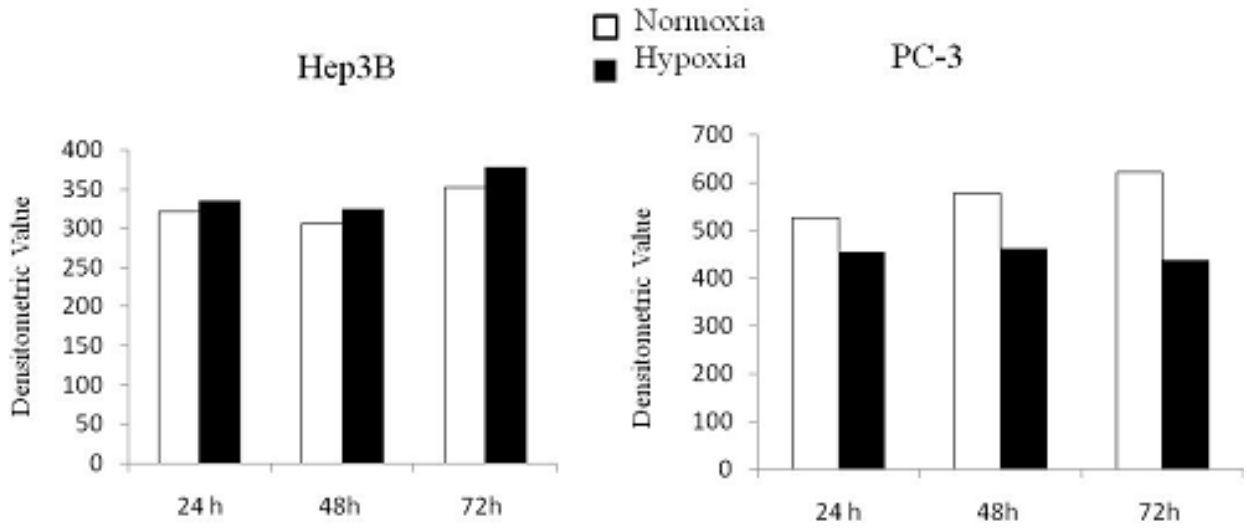

Figure 3. (a) Expression of β-2 Microglobulin in Hep3B cell line (a) and PC3 cell line (b) in normal and hypoxic conditions.

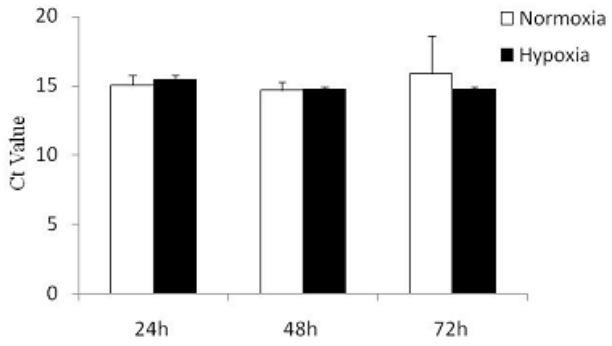

Figure 4. Expression of β-2 Microglobulin in Hep3B cell line in normal and hypoxic conditions (Real Time PCR).

DISCUSSIONS

Selection of internal control in RNA analysis is very important. When studying different working conditions investigators have to choose internal control which doesn't affected their working conditions. Hypoxia is a critical stimulus in many physiological and disease states. Cells respond to hypoxia by regulating the expression of a number of genes, including erythropoietin, vascular endothelial growth factor, and various glycolytic enzymes. This regulation is mediated in part by transcription factors of the hypoxia-inducible factor HIF-1 family [7]. Mechanisms of sensing and adaptation to low oxygen have been extensively studied in the mammalian system. In mammalian systems, cobalt chloride ($CoCl_2$) has been used as a chemical agent that reportedly induces a biochemical and molecular response similar to that observed under low-oxygen conditions [9-14]. It has been well documented that cobalt, a transition metal, mimics hypoxia by causing the stabilization of HIF-α. A recent model suggested that the hydroxylation of HIF-α is mediated by a group of HIF-specific hydroxylases and that cobalt can inactivate the enzymes by occupying an iron-binding site on the proline hydroxylases.

Recently, studies showed that several genes normally used as reference genes had varying expression levels in hypoxic conditions. In our study we examined different internal control genes which can be affected by chemical hypoxia in Hep3B and PC-3 cell lines.

In 1999, Hue Zeng *et al*. tested GAPDH, β-actin, cyclophilin and 28S rRNA under hypoxia compared to normoxia and compared RNA levels of these 4 housekeeping genes using ribonuclease protection assays. They showed that 28S rRNA is a reliable internal control for comparative analyses of transcription under hypoxia; GAPDH appears particularly unfavorable for this purpose either in hypoxia or other experimental conditions that upregulate HIF-1α [1]. Several investigators reported that GAPDH expression is regulated by a variety of factors like calcium [15], insulin [16] and hypoxia [17]. The degree to which hypoxia transcriptionally activates GAPDH is cell- type specific [8, 17-19].

In 2007, Harun Said et al. analyzed the degree of regulation of GAPDH expression in human glioblastoma cells under hypoxic conditions *in vitro* in comparison to other housekeeping genes like β-actin, serving as experimental loading controls. They showed that GAPDH an optimal choice of a housekeeping gene and/or loading control to determine the expression of hypoxia induced genes at least in glioblastoma [8]. In this study, they also summarized the literature which is related with internal controls. They showed that GAPDH overexpressed in LNCap, ATII, SiHa, MBEC4, EC4 and Rabbits skeletal muscle cells under hypoxic conditions [8, 17, 19-28]. Our results also showed that mRNA level of GAPDH is increasing in time dependent manner in Hep3B cells..

In 1998, Trine *et. al* compared the gene expression levels of a panel of twelve widely used reference genes during hypoxic culture, osteogenic and chondrogenic differentiation, and passaging of primary human ASCs. They showed that 18S rRNA, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and beta-actin were unsuitable for normalization in the conditions they tested, whereas tyrosine 3/tryptophan 5-monooxygenase activation protein (YMHAZ), TATAA-box binding protein (TBP), beta-glucuronidase (GUSB) were the

most stable across all conditions [29]. Our results also showed that mRNA level of GAPDH and β-Actin affected by $CoCl_2$-induced hypoxic conditions. β-2 Microglobulin mRNA level remain more stable in Hep3B and PC3 cell lines. Therefore, it could be concluded that β-2 Microglobulin can be used as an internal control in chemically induced hypoxic conditions.

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