The International Journal of Biochemistry & Cell Biology 65 (2015) 169–181

Contents lists available at ScienceDirect

The International Journal of Biochemistry & Cell Biology

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

Heparin treatment increases thioredoxin interacting protein expression in hepatocellular carcinoma cells

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ARTICLE INFO

Article history:
Received 1 January 2015
Received in revised form 30 April 2015
Accepted 28 May 2015
Available online 30 May 2015

Keywords:
Heparin
HCC
TXNIP
ChoRE
ChoRE associated proteins

ABSTRACT

Heparins play an important role in cell growth, differentiation, migration and invasion. However, the molecular mechanisms of heparin mediated cellular behaviors are not well defined. To determine the effect of heparin on gene expression, we performed a cDNA microarray in a hepatocellular carcinoma cell line and found that heparin regulates transcription of genes involved in glucose metabolism.

In this study, we showed a new role of heparin in the regulation of thioredoxin interacting protein, which is a major regulator of glucose metabolism, in hepatocellular carcinoma cell lines. We determined the importance of a unique carbohydrate response element located on its promoter for the heparin-induced activation of thioredoxin-interacting protein and the modulatory role of heparin on nuclear accumulation of carbohydrate response element associated proteins. We showed the importance of heparin mediated histone modifications and down-regulation of Enhancer of zeste 2 polycomb repressive complex 2 expression for heparin mediated overexpression of thioredoxin-interacting protein. When we tested biological significance of these data; we observed that cells overexpressing thioredoxin-interacting protein are less adhesive and proliferative, however they have a higher migration and invasion ability. Interestingly, heparin treatment increased thioredoxin-interacting protein expression in liver of diabetic rats.

In conclusion, our results show that heparin activates thioredoxin-interacting protein expression in liver and hepatocellular carcinoma cells and provide the first evidences of regulatory roles of heparin on carbohydrate response element associated factors. This study will contribute future understanding of the effect of heparin on glucose metabolism and glucose independent overexpression of thioredoxin-interacting protein during hepatocarcinogenesis.

1. Introduction

Thioredoxin interacting protein (TXNIP), a major regulator of cellular stress conditions, is a negative regulator of Thioredoxin (TRX) and it has important roles in several biological responses; such as cellular viability, vascular invasion and angiogenesis (Chen et al., 2008; Kim et al., 2007; Parikh et al., 2007). TXNIP has been described as a regulator of glucose metabolism (Parikh et al., 2007) and it has been reported that glucose elevates TXNIP gene expression level (Muoio, 2007).

Upregulation of TXNIP expression in response to high glucose is mediated by unique non-palindromic E-box (CACGTG) repeats that function as Carbohydrate response element (ChoRE). The Mondo family basic helix-loop-helix transcription factors: Carbohydrate-responsive element-binding protein (ChREBP) and Mlx interacting protein (MondoA), with their common binding partner Max-like protein X (Mlx) are key sensors of intracellular glucose level in mammals. ChREBP and MondoA are the most important regulators of glucose induced transcriptional responses and have differential tissue specific roles in mammals. ChREBP is expressed predominantly in the liver, adipose tissue and pancreatic beta cells (Dentin et al., 2006; Wang and Wollheim, 2002; Yamashita et al., 2001) whereas MondoA predominantly expressed in skeletal muscle and up-regulates glycolytic target genes (Billin et al., 2000; Havula and Hietakangas, 2012). The ChREBP/MondoA-Mlx complex is activated by glucose, and regulates expression of target genes that contains

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http://dx.doi.org/10.1016/j.biocel.2015.05.025
1357-2725/

a ChoRE in their promoter region (Chutkow et al., 2008; Ma et al., 2006; Minn et al., 2005).

Previous studies reported a two-step mechanism for glucose-mediated activation of ChREBP. Phosphorylation of a Ser196 residue, which is located close to nuclear localization signal and DNA binding sequences and, a Thr666 residue, which is located close to transcriptional activation domain, both ensures the cytoplasmic localization of the protein. Following glucose stimulation, phosphatase 2A (PP2A) is activated and dephosphorylates Ser196 residue of ChREBP and hence facilitates its nuclear transport. Heterodimerization with Mlx is required for nuclear localization of MondoA and ChREBP (Peterson et al., 2010). Dephosphorylation of Thr666 residue in ChREBP occurs in nucleus to maintain the activation (Kawaguchi et al., 2001).

TXNIP functions downstream of MondoA/ChREBP-Mlx and negatively regulates glucose uptake when intracellular glucose concentration is exceedingly high (Cha-Molstad et al., 2009; Stoltzman et al., 2008). Recent data shows that MondoA-Mlx complex cannot stably bind TXNIP promoters and immediately exported from nucleus in low glucose conditions. However in the presence of high glucose MondoA-Mlx complex binds to the ChoRE and recruits transcriptional co-activators and activates TXNIP expression. Peterson et al. (2010) reported that, glucose stimulates recruitment of histone modifying enzymes to promoter bound MondoA-Mlx to trigger epigenetic modifications that activates TXNIP gene expression. In addition to the importance of H3K27me3, histone deacetylation in the regulation of TXNIP gene is reported in leukemia model (Zhou et al., 2011).

Although several studies focused on revealing molecular mechanisms of glucose dependent TXNIP expression, recent studies indicated that regulation of TXNIP expression might be glucose independent (Han et al., 2003; Junn et al., 2000). Glucose independent TXNIP activation inducers are not well defined yet.

Heparin is a linear, unbranched, highly sulfated glycosaminoglycan (GAG). It is known to be the most negatively charged molecule due to its high content of *-sulfo* and *-carboxyl* groups (Gandhi and Mancera, 2008). In addition to its anticoagulant capacity, heparin also interacts with a wide range of proteins to modulate their activities. These interactions are important for the regulation of many biological processes, such as cell growth and differentiation, cellular morphogenesis and signaling, cell–cell and cell-extracellular matrix (ECM) interactions and inflammation (Mulloy and Forster, 2000). However, the molecular mechanism behind these effects has not been determined yet.

In our previous studies, we showed the effects of heparin on hepatocyte growth factor induced motility and invasion in hepatocellular carcinoma (HCC) cells (Ozen et al., 2012). In order to identify the molecular mechanisms of heparin mediated biological responses, we performed a gene expression array with a HCC cell line. The microarray analysis revealed that heparin mediates transcription of multiple genes involved in glucose metabolism. TXNIP was one of the most significant differentially expressed genes.

In this study, we first tested the dose and time dependent effects of heparin on TXNIP expression in HCC cell lines. Then we examined *in vivo* effects of heparin on TXNIP expression in liver, by using rat diabetes model. Afterwards, we tested the hypothesis that whether heparin affects TXNIP gene expression *via* a similar molecular mechanism to glucose. Since TXNIP expression is mediated by shuttling of ChREBP/MondoA and Mlx proteins from cytoplasm to nucleus, we next examined the nuclear accumulation of these proteins following heparin treatment. To better understand the role of heparin in the regulation of TXNIP expression, histone modifications that are related to activation of TXNIP gene were examined. Since TXNIP is known as a target of Enhancer of zeste homolog 2 (EZH2), which is known to be an important component of the Polycomb Repressive Complex (PRC2), the effect of heparin on EZH2 expression and activation was studied. To understand the biological significance of heparin mediated TXNIP expression, we evaluated the effect of TXNIP overexpression on adhesion, proliferation, motility and invasion of HCC cells by overexpression studies. Therefore, our study presents a novel model for the role of heparin in the regulation of TXNIP expression, *via* ChoRE and associated proteins in HCC cell lines.

2. Materials and methods

2.1. Cell culture

Human HCC cell lines; HuH-7, PLC/PRF/5, SNU-449 and SK-HEP-1, were cultivated as described previously (Bozkaya et al., 2012). Cell line authentication was performed by Short Tandem Repeat (STR) analysis at the University of Colorado Cancer Center (UCCC) DNA Sequencing & Analysis Shared Resource (CO, USA) using Applied Biosystem's Identifier kit (PN 4322288). Cells were treated with different concentrations (1, 10, 100 μg/ml) of heparin at indicated time points after 16 h serum starvation in DMEM with 2% FBS. Cells were treated with 5 mM and 22.5 mM glucose after 16 h glucose starvation in DMEM with 0% glucose. In preliminary studies, the effects of different heparins (Applichem A3004, Biochrom L651, Calbiochem 3750973, *etc.*) were tested and similar results were observed with all types of heparins.

2.2. Generation of stable cell lines

HuH-7 cells were transfected with p-CMV-6AC-MOCK and p-CMV-6AC-TXNIP plasmids by using Fugene HD Transfection Reagent (Promega E2312), according to manufacturer's instructions. Transfected cells were selected in medium containing 400 μg/ml Geneticin (Life Technology 10131-027). TXNIP overexpression was confirmed by Western blotting (WB).

HuH-7 cells were stable co-transfected with lentiviral plasmids pGIPZ-EZH2 shRNA mir V2LHS-17510 and pGIPZ-EZH2 shRNA mir V2LHS-63068 (Thermo Scientific) for silencing EZH2 by using Trans-Lentiviral Packaging Kit (Thermo Scientific). Plasmid containing scrambled shRNA was used as a negative control. Cells were maintained in selection growth medium with 4 μg/ml puromicin antibiotic (Life technologies A1113803) following transfection. EZH2-silenced and scramble control HuH-7 cells were obtained and confirmed by qPCR and western blotting.

2.3. Animal studies

Total 37 adult, male Wistar rats weighing 200–250 g were used in this study. All procedures involving animal studies were reviewed and approved by the "Ethics Committee of Research of Laboratory Animals" of Dokuz Eylul University Medical School (Protocol number: 2010/58) and performed in accordance with "Guide for the Care and Use of Laboratory Animals of the National Institutes of Health" regulations. All animals were kept in standardized conditions of temperature (21–22 °C) and illumination (12:12 light/dark) and cages with mesh bottoms providing free access to tap water and pelleted food. The animals were not fed for 12 h before the experiment, but had free access to water until the beginning of the experiment.

2.3.1. Experimental study design

Rats were divided into four groups as follows: STZ-induced diabetic rats (D, $n = 8$), age matched control rats (C, $n = 5$) and insulin-treated diabetic rats (D + I, $n = 8$) and 3 mg/kg low molecular weight heparin (LMWH) treated diabetic rats (D + H). While the