INVOLVEMENT OF AMINOLEVULINIC ACID SYNTHETASE (ALAS) IN THE HEPATIC GLYCOGEN DEPLETION CAUSED BY DIOXIN EXPOSURE

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Abstract

Administration of a lethal dose of 2,3,7,8-teterachlorodibenzo-*p*-dioxin (TCDD) to laboratory animals results in depletion in glycogen in the liver and a marked decrease in body weight, called wasting syndrome. Epidemiologic studies suggested that an association of dioxin exposure with an occurrence of type 2 diabetic mellitus symptoms, such as in Vietnam War veterans. Therefore a possibility of disruption of glucose metabolism by dioxin has been suspected. Here, we studied whether glycogen is decreased by TCDD exposure in the liver cells. When three hepatoma cell lines, HepG2 (human), Hepa1c1c7 (mouse), and H4IIE (rat) were exposed to 1 to 100 nM TCDD, glycogen amounts in the cells were decreased in a dose-dependent manner. Next, because microarray analysis showed that exposure to TCDD induced ALAS mRNA in the female mouse liver in vivo and HepG2 in vitro, we examined a possible involvement of aminolevulinic acid synthetase (ALAS), the rate limiting enzyme of heme synthesis, in this glycogen reduction. The transfection of ALAS specific siRNA suppressed a TCDD-induced decrease in glycogen amounts in the HepG2. In conclusion, TCDD causes decrease in glycogen in hepatic cells *in vitro*, and ALAS is involved in a TCDD-induced decrease in the glycogen amounts.

Introduction

Administration of a lethal dose of 2,3,7,8-teterachlorodibenzo-*p*-dioxin (TCDD) to laboratory animals results in a marked persistent decrease in body weight, leading to death. This disease state, known as wasting syndrome is a typical adverse toxicity of TCDD. Because no such effects were observed in AhR-null mice, the wasting syndrome is thought to be caused by AhR-dependent mechanism[1]. However, the pathogenesis of this syndrome is still obscure. One possibility is that the glucose metabolism affected by TCDD plays a significant role. This supposition is based upon a few lines of experimental evidence that a lethal dose of TCDD caused depletion in glycogen in the liver and plasma[2, 3], and repressed the expression of phosphoenolpyruvate carboxykinase (PEPCK), a rate-limiting enzyme in gluconeogenesis[4]. In addition, TCDD is suggested to be involved in diabetic mellitus by epidemiologic studies on the Vietnam War veterans who had been engaged in spraying defoliants[5]. *In vivo* experiments also suggest that TCDD reduced the secretion of insulin from islets[6]. Taken together, TCDD is thought to disrupt glucose homeostasis. In the present study, to elucidate the molecular mechanism of TCDD-induced wasting syndrome, we developed an *in vitro* model using hepatoma cell lines in which glycogen contents in the cell was reduced by TCDD exposure.

Materials and method

Cell culture and chemical exposure: Human hepatoma cell lines (HepG2), mouse hepatoma cell line Hepa1c1c7 and rat hepatoma cell line H4IIE were obtained from RIKEN Cell Bank (Tsukuba, Japan). HepG2 was grown in DMEM as a base medium, supplemented with 10% inactivated fetal bovine serum, 1% penicillin /streptomycin, and 0.1% 2-mercaptoethanol. These cells were cultured in a humidified air incubator supplemented with 5% CO_2 at 37°C. TCDD exposure was performed after the 24 hr incubation in serum- and glucose-free medium. A vehicle (dimethylsulfoxide, DMSO) concentration was kept identical in culture medium at 0.1% (v/v).

Transfection of siRNA: ALAS siRNA was purchased from Ambion (Austin, USA). Cells were suspended in normal growth medium to 2.7×10^5 cells/ml. 75 pmol of siRNA and 30 µl of siPORT NeoFX Transfection Agent (Ambion) diluted in 1 ml of Opti-MEM was added to the 5 ml of cell suspension and then plated onto 10 cm diameter dish. After 8 hr incubation, the medium was changed to 10 ml of normal growth medium. Serum- and glucose-free medium was then replaced 24 hr after the transfection.

Glycogen assay: Determination of glycogen was performed using a modified protocol of phenol-sulfuric acid colorimetric method[7, 8]. Cells were scrapped in 1 ml potassium hydroxide (30%) saturated with Na_2SO_4. The homogenate was incubated in a boiling water bath for 1 hr to stop enzyme reactions. These samples were kept on ice for 5 min, followed by an addition of one volume of ice-cold 95% ethanol, and kept at 4°C overnight to precipitate glycogen. After centrifugation at 1,000 × *g* for 30 min, the glycogen precipitates were dissolved in 1ml of water, followed by the addition of 1ml of 5% phenol and 5ml of H_2SO_4. After incubation at 30°C for 15 min, glycogen amounts were quantified spectroscopically with an absorbance at 490 nm, and were normalized by cell number.

Real-time RT-PCR: Total RNA was extracted from cells using RNeasy mini kit (QIAGEN, Hilden, Germany). This RNA sample was reverse-transcribed using SuperScript III RNase H-Reverse Transcriptase and Oligo (dT)12-18 Primer (Invitrogen, Carlsbad, USA). Quantitative real-time RT-PCR was performed using a Light Cycler rapid thermal cycler system (Roche Molecular Systems, Alameda, USA) with SYBR Premix Ex Taq (Perfect Real Time) (Takara Bio, Otsu, Japan). To draw a standard curve, a series of dilution-specific PCR products that were prepared in advance and purified using a QIAquick PCR Purification kit (QIAGEN) were used. Shuttle PCR (40 cycles) was conducted in glass capillaries (Roche) under the following condition: denaturation at 95°C for 10 sec, and extension at 64°C for 30 sec. Fluorescent products were detected at the end of the 64°C extension period, and the specificity of the amplified PCR products was confirmed by melting curve and 3% agarose gel electrophoresis analysis. All quantitative data were calculated by dividing the copy number of targets by the original RNA concentration.

Western blotting: Cells were homogenized with sample buffer containing 62.5 mM Tris-HCl, pH6.8, 10% glycerol, 2% SDS and 5% 2-mercaptoethanol. Proteins were size-fractionated on a 10% SDS-PAGE gel. Rabbit polyclonal antibodies for ALAS (Abcam, Cambridge, UK) and mouse monoclonal antibodies for β–actin (SIGMA, St. Louis, USA) were used for immunoblot staining. Goat anti-rabbit polyclonal antibody horseradish

peroxidase-conjugated (PIERCE, Rockford, USA) and goat anti-mouse polyclonal antibody horseradish peroxidase-conjugated (Invitrogen) were used as the secondary antibodies.

Results and discussion

Glycogen amounts in hepatoma cells were decreased by TCDD exposure.

Administration of a lethal dose of TCDD caused depletion of glycogen in the liver and plasma. However, any *in vitro* model to mimic this phenomenon has not been reported. Therefore, we determined amounts of glycogen in HepG2 and evaluated the effects of TCDD exposure. In this study, we precultured cells in serum- and glucose-free medium from 24 hr before TCDD exposure because glucose uptake from the normal growth medium may affect the glycogen depletion. When HepG2 was exposed to 1, 10 and 100 nM TCDD for 24 hr, the glycogen amounts in the cells were decreased in a dose-dependent manner, at 95, 60 and 20% of the control cells respectively. A decrease in glycogen by TCDD was observed in mouse hepatoma cell line Hepa1c1c7 and rat hepatoma cell line H4IIE. Thus, these data suggest that TCDD decreased glycogen amounts by directly acting on liver cells.

ALAS mRNA is involved in TCDD-induced glycogen reduction.

By using microarray, we found that ALAS mRNA was dose-dependently induced by TCDD exposure in three strains of mouse liver, BALB/cAN, C3H/He and CBA/JN. Then we analyzed possible TCDD effects on the expression of ALAS mRNA and protein using the HepG2 *in vitro* model. Real-time RT-PCR and Western blotting analyses revealed that TCDD induced the ALAS expression at the mRNA (1.53-fold) and protein (1.43-fold) level in this model. This result was consistent with the one obatained by microarray analysis.

We next investigated whether the enzyme activity of ALAS affects the glycogen metabolism in the HepG2 *in vitro* model. ALAS is a rate-limiting enzyme in the heme biosynthetic pathway. Because the porphyrias are disorders, each involving a specific enzyme, in the heme biosynthesis pathway and glucose treatment ameliorated the hepatic porphyria[9], and because patients afflicted with porphyria were accompanied with hyperinsulinemia[10], the heme synthesis pathway is presumably linked to glucose metabolism. In addition, it was reported that ALAS transcription is regulated by insulin in *in vivo* and *in vitro*[11, 12]. We determined amounts of glycogen in HepG2 cells that were trasfected with ALAS siRNA. In these cells, ALAS mRNA and protein expression levels were decreased to 44% and 74% of the control level respectively, and the suppression by ALAS siRNA was not affected by TCDD treatment. When these ALAS siRNA-transfected cells were exposed to 1, 10 and 100 nM TCDD for 24 hr, no significant difference in glycogen amounts was found between the TCDD-treated cells and control cells. These date suggested that ALAS is involved in a TCDD-induced decrease in glycogen amounts.

In the present study, ALAS expression was increased by TCDD at the mRNA and protein level. Because ALAS promoter has 10 xenobiotic response elements (XRE) in the 5'-flanking transcriptional regulatory region and 17 in the internal genomic sequence, there may be the possibility that AhR ligands may transactivate ALAS gene by an XRE-dependent mechanism. On the other hand, ALAS mRNA level was stimulated with a reciprocal relation with the amounts of heme in the cell, and vice versa by negative feedback[13]. TCDD was reported to decrease the amounts of heme molecules in the rat testis[14], and sub-lethal dose of TCDD administration resulted

in the onset of porphyria and the suppression of activity of enzymes in the heme biosynthetic pathway, such as uroporphyrinogen decarboxylase[15, 16]. Therefore, an increase in the ALAS transcription found in TCDD-exposed cells is speculated to be induced by a decrease in the amounts of heme by TCDD treatment. Whether up-regulation of ALAS transcription is regulated by TCDD-AhR transactivation or negative feedback of heme reduction should remain investigated.

In summary, we developed *in vitro* model of glycogen reduction in the cell by TCDD exposure using hepatoma cell lines, and revealed that ALAS is involved in this phenomenon. Our model would be useful for analyzing the mechanism of glycogen reduction of dioxin-induced wasting syndrome and the interaction of heme biosynthetic pathway and glucose metabolism.

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