METALLOTHIONEIN AS A MARKER OF TCDD-INDUCED OXIDATIVE STRESS IN THE RAT

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Introduction

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is a prototype for environmental agonists of the aryl hydrocarbon receptor (AhR) that are known to produce multiple adverse effects in human as well as laboratory animals. Although not directly genotoxic, dioxin is known to increase transformation and mutations in mammalian cells in culture and to cause an exaggerated oxidative stress response in the female rat[1]. It is reported that oxidative stress following TCDD administration is AhR-mediated[2]. One proposed pathway for that is through the metabolic activation of estrogen via TCDD-induced cytochrome P4501A1 (CYP1A1) and CYP1B1 enzymes[3]. Oxidative stress may play an important role in toxicity of TCDD. On the other hand, metallothionein (MT) is a cysteine-rich metal-binding protein that plays a detoxification role against harmful heavy metals such as cadmium (Cd) and mercury. MT also suggested to maintain intracellular homeostasis of essential metals like copper and zinc[4]. MT has been thought to be involved as a free radical scavenger in cooperation with established biomolecules that act as antioxidants, such as reduced form of GSH. MT protein or mRNA induction in experimental animals and human after ionizing radiation or UV radiation has been reported *in vitro* and *in vivo* stuides[5]. MT induction was found to protect oxidative damage after ionizing radiatin[6]. Pre-treatment of Cd, a potent inducer for MT synthesis, was confirmed to protect skin damage by UV radiation *in vitro*, suggesting a protective role of MT against UV-induced oxidative stress in skin[7]. We showed previously that an increase in levels of MT by treatment of dexamethasone and zinc produced a resistance of skin cells to UVB irradiation[8]. Since little attentions have been paid to the putative antioxidative functions of MT in TCDD-exposed animals, the aim of the present study was to evaluate the possible involvement of MT as an antioxidants in oxidative responses induced by TCDD.

Materials and Methods

Animals and treatments: Six-week-old female Sprague-Dawley (SD) rats were given a single oral dose of TCDD (1.0, 2.0 or 4.0 μg TCDD/kg body weight) or equivalent volume of corn oil as a vehicle. Seven days after TCDD administration, blood was collected from six rats of each group and serum was processed. The left lobes of liver were fixed in 10% buffered formalin for the immunohistochemical studies. The remainder of the liver was removed and processed immediately for RNA extraction.

Immunohistochemistry: Liver sections were stained for MT and HO-1 by an indirect immunohistochemical technique[9]. The primary antibody was replaced with normal rabbit IgG as negative controls.

MT and Metal Analysis: Hepatic MT contents in the liver were determined by radioimmunoassay as described earlier[10]. Copper (Cu), zinc (Zn) and iron (Fe) concentrations in the liver were analyzed by inductively coupled plasma emission spectrometry (ICP) after digesting samples with an acid mixture (HNO_3:$HClO_4$= 3:1).

RNA Extraction and RT-PCR: Total hepatic RNA was extracted by using Isogen (Nippon Gene, Tokyo, Japan). PCR primers for amplification of CYP1A1 and β-actin were described by Vanden Heuvel et al. [11]. PCR primers for MT-I and MT-II and methods of the first-strand cDNA synthesis for RT-PCR were performed by the procedure described earlier[12].

Cytokine Analysis: Serum levels of IL-1β, IL-6, TNF-α and IL-10 were quantified with Biotrac ELISA kits (Amersham Life Science, Buckinghamshire, UK), according to the manufacturer's instructions.

Determination of 8-OHdG and GSH: Serum 8-OHdG levels were measured with ELISA kit (Japan Institute for Control of Aging, Fukuroi, Japan) according to the manufacturer's instructions. Reduced glutathione (GSH) concentration in the liver was determined using Cayman's GSH assay kit (Cayman Chemical Company, Ann Arbor, Michigan USA) according to the manufacturer's instructions.

TCDD Analysis: Livers, adipose tissues and serum were analyzed for TCDD concentration by using gas chromatography-mass spectrometry procedures with specific ion monitoring. Quantified values were calculated by the internal standard methods.

Statistical Analysis: Differences in means among the 4 groups were analyzed by one-way ANOVA with a post-hoc Fisher's PLSD method and statistically significance was set at $p< 0.05$.

Results and Discussion

The highest level of TCDD was found to accumulate in the liver followed by adipose tissue. Amounts of TCDD in the liver dosed with 1.0, 2.0 and 4.0 μg/kg bw were 6.0, 12.6 and 28.9 ng/g tissue, respectively. The expression of CYP1A1 was significantly induced by TCDD at a dose as low as 1 μg/kg bw in a dose-

dependent manner.

TCDD was found to dramatically induce MT production in the liver by doses as low as 1.0 μg TCDD/kg in a dose-dependent fashion. The highest MT concentration attained at a dose of 4.0 μg TCDD/kg was 407 μg/g tissue, which is approximately 10-fold of MT concentration in the control rat liver. Expression of MT-I and II genes in the liver were semiquantified by RT-PCR. MT-I and II genes were induced markedly in a dose-dependent manner, which was in good agreement with the MT amounts in the liver. While nearly no immunostaining for MT was found in control rat liver, it was clearly shown that TCDD increased not only immunostainability, but also numbers of MT-positive cells.

We next investigated changes in localization of heme oxygenase-1, one of the oxidative stress-responsible enzymes, during TCDD-induced oxidative stress. While no immunostaining for heme oxygenase-1 protein was found in control rat liver, TCDD (2.0 and 4.0 μg/kg bw) resulted in appearance of heme oxygenase-positive cells in hepatic macrophages (Kupffer cells).

A dose-response effects of TCDD on concentrations of Cu, Zn or Fe in the liver was investigated since Cu and Fe are involved in the cellular redox system and Cu and Zn were MT inducers. Among those metals, a dose-dependent increase in Fe concentration was noted, almost 1.6-fold higher than control at the highest dose-group. TCDD treatment at doses as low as 1.0 μg TCDD/kg also produced significant increase in Cu levels, while Zn levels were increased significantly at the highest dosed-group (4.0 μg/kg bw).

Serum levels of 8-OHdG, a sensitive marker for DNA damage by reactive oxygen species, were quantified. Serum 8-OHdG level of the control rats was 2.8 ng/ml. TCDD dose at 2.0 μg/kg bw and 4.0 μg/kg bw increased serum 8-OHdG concentrations to 2-fold and 2.5-fold, respectively, indicating production of oxidative stress-mediated DNA damage. Significant decrease in GSH levels were observed in the liver of the rats treated with 4.0 μg TCDD/kg bw. Because macrophage-derived cytokines have been shown to be involved in the production of active oxidative species and expression of MT genes, serum levels of IL-1ß, IL-6, IL-10 and TNF-α were measured in TCDD-exposed rats. TCDD did not affect any of the cytokines measured in this experiment.

In this paper, we showed that a low-dose single TCDD exposure produced an oxidative stress in rats by measuring oxidative stress markers, such as an increased expression of CYP1A1, an enhancement of 8-OHdG levels, a decrease in hepatic GSH levels and an induction of heme oxygenase-1 activity. In addition, we demonstrated that TCDD at a dose of 1.0 μg/kg bw is low enough to induce MT and mRNA, the liver TCDD concentration was 6.03 ng/g tissue. In contrast, hepatic levels of serum 8-OHdG and the reduced form of GSH were altered at 2.0 and 4.0 μg TCDD/kg bw, respectively. Although the toxicological significance of the elevated level of MT is not clearly understood, the increase is one of the earliest biomarker so far reported.

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